Arch Pharm Res Vol 25, No 5, 572-584, 2002

Archives of Pharmacal Research
http://apr.psk.or.kr

Protein Drug Oral Delivery: The Recent Progress

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(Received April 30, 2002)

Rapid development in molecular biology and recent advancement in recombinant technology increase identification and commercialization of potential protein drugs. Traditional forms of administrations for the peptide and protein drugs often rely on their parenteral injection, since the bioavailability of these therapeutic agents is poor when administered nonparenterally. Tremendous efforts by numerous investigators in the world have been put to improve protein formulations and as a result, a few successful formulations have been developed including sustained-release human growth hormone. For a promising protein delivery technology, efficacy and safety are the first requirement to meet. However, these systems still require periodic injection and increase the incidence of patient compliance. The development of an oral dosage form that improves the absorption of peptide and especially protein drugs is the most desirable formulation but one of the greatest challenges in the pharmaceutical field. The major barriers to developing oral formulations for peptides and proteins are metabolic enzymes and impermeable mucosal tissues in the intestine. Furthermore, chemical and conformational instability of protein drugs is not a small issue in protein pharmaceuticals. Conventional pharmaceutical approaches to address these barriers, which have been successful with traditional organic drug molecules, have not been effective for peptide and protein formulations. It is likely that effective oral formulations for peptides and proteins will remain highly compound specific. A number of innovative oral drug delivery approaches have been recently developed, including the drug entrapment within small vesicles or their passage through the intestinal paracellular pathway. This review provides a summary of the novel approaches currently in progress in the protein oral delivery followed by factors affecting protein oral absorption.

Key words: Protein oral delivery, Absorption site, Metabolism, Permeability, Transporters, Solubility, Molecular size, Innovative pharmaceutical approaches, Microencapsulation, Nanoparticles, Mucoadhesion, Transport vectors

INTRODUCTION

Perhaps, the questions for a pharmaceutical scientist who himself does not have clear answers, are Can proteins be delivered orally? and If so, how and when can we have a product? Before frustrated, we should look into the fact that many remarkable medicines in the present market were even unthinkable projects not too long ago.

Currently, over 100 peptide and protein drug products are under clinical investigation and about 30 compounds got FDA approval (Scrip's report 2001). This is an astonishing progress and could be attributed to the rapid development on recombinant DNA techniques and biotechnology. The dosage forms for peptide and protein drugs rely on their parenteral injection. Among the therapeutic peptide and protein drugs currently available as parenterals, insulin, human growth hormone (hGH), calcitonin, vasopressin, somatostatin, growth factors, leuprolide, erythropoietin (EPO), and cytokins that require frequent administration, are urging pharmaceutical scientists to develop more convenient dosage forms for noninvasive administration. An oral dosage form is the most preferable one because of the accompanying advantages of patient comfort, ease of administration, decreased medical costs, and reduced patient compliance.

Significant efforts in academic and commercial laboratories have been put but major breakthroughs in oral peptide and protein formulation achieving predictable and reproducible drug profiles in therapeutic doses without wasting up to 99% of the drug, have not been achieved

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(Cleland *et al.*, 2001; and Vyas *et al.*, 1997). The gastrointestinal system is designed by nature to digest proteins and to absorb the digested protein fragments, e.g., amino acids or di- or tripeptides. Therefore, it is not difficult to imagine the hurdles for scientists to overcome for successful protein drug oral delivery. The major barriers (Fix, 1996) in developing oral formulations for peptides and proteins include poor intrinsic permeability, luminal and cellular enzymatic degradation, rapid clearance, and chemical and conformational stability. Conventional approaches to address these barriers, which have been workable with traditional, small, organic drug molecules, have not readily translated into effective peptide and protein formulations (Cleland *et al.*, 2001).

This review will describe a brief summary on the overview of the existing approaches, the recent advances that have been made in oral delivery as related to proteins, and some future directions to consider followed by reviewing factors affecting oral protein absorption. Several excellent review papers have been written in lieu of protein oral delivery (DiBiase and Morrel, 1997; Fasano, 1998a & b; Fix, 1996; Kompella and Lee, 2001; Lee 1991; Lehr, 1994; Leone-Bay *et al*, 2000; Merkle, 1994 Sakuma *et al*, 2001; Vyas *et al.*, 1997; Woodley, 1994). Therefore, detailed description on individual technologies that have been described in elsewhere would not be covered in this review. It is likely that effective oral formulations for peptides and proteins will remain highly compound specific, but principle concept to preserve protein integrity before its reach to systemic circulation would be the same.

FACTORS AFFECTING ORAL PROTEIN ABSORPTION

Absorption of peptides (greater than three or four residues) and proteins by epithelial cells is achieved by the process of receptor-mediated or non-receptor-mediated endocytosis (also called pinocytosis, when the material taken up is entirely soluble) and this is the most likely route of uptake and possible translocation of an orally administered therapeutic protein. Recent findings on many transporters (Yang *et al.*, 1999; Lee, 2000) add a transporter-mediated uptake as a possible absorption mechanism for proteins in addition to paracellular absorption mechanism. Oral delivery of particulates in which the protein drugs were embedded to be protected from the gut enzymes, may require another understanding in uptake and or absorption. Many groups believe that uptake of these particulates is a function carried out by membraneous/microfold (M) cells in Peyers patches of the mammalian gut. However, other groups claim that uptake across the villous epithelium occurs, via damaged regions or by a paracellular route (Kreuter, 1991; OHagan, 1994). Thomas *et al* (1996) found that the absorption of microparticles across the Peyers patches of rats, mice and rabbits can occur rapidly as microparticles can be detected in the lymph soon after intestinal delivery. Detailed reviews on the absorption mechanisms for protein drugs following oral administration are available in the literature (DiBiase and Morrel, 1997; Tsuji and Tamai, 1996).

Protein-base drugs are by nature disintegrated by various enzyme systems before its reach to the systemic blood circulation. Once it is protected from enzymatic degradation by any means, large molecular size together with hydrophilic property of proteins can be another stumbling block for the intestinal membrane transfer. Systematic yet mechanistic understanding on the intestinal absorption of protein drugs would be essential to circumvent the fundamental hurdles in protein oral delivery. In parallel, integration of sub-cellular mechanisms into in vivo effect would be necessary to set a priority in developing a strategy for protein drug oral delivery. Numerous research papers during the last decade described factors affecting low oral bioavailability of protein drugs (Fasano, 1998a, b, &c; Kompella and Lee, 2001; Woodley, 1994). The most advanced findings are summarized below. Effective and successful oral protein delivery requires further development in understanding these complex mechanisms in order to maximize the therapeutic potential of this class of compounds.

Absorption site

In addition to metabolic differences, the anatomical, physiological, and biochemical differences between the various part of GI tract can cause significant variation in protein drug oral absorption (Garrido, 1981; Kararli, 1995). Among the physiological factors, regional differences in composition and thickness of mucus, pH, surface area, enzyme activity, bile, pancreatic juice, mucus and fluid volume and content can modify dissolution rates, solubility, transit times, metabolism, and membrane transport of the drug molecules. The lipid/protein composition of the enterocyte membrane along the GI tract can alter binding and transport of drugs. The location and number of Peyer's patches, which are most abundant in the ileum, can also be important in the absorption of large molecules and particulate matters. Consequently, the existence of (better) absorption window could not be a new discovery for peptide and protein drugs administered orally. For peptides, cyclosporine (Grevel *et al.*, 1986) and desmopressin (Lundin and Vilhardt, 1986) exhibited maximum absorption in the duodenum in humans and rabbits, respectively, while octreotide, a somatostatin analog, in the lower GI tract in humans (Kohler *et al.*, 1987). Although little information exists on site-to-site variation in the protein absorption, the

absorptive cells of neonatal rats have been shown to capture nerve growth factor (Owen and Bhalla, 1983), epidermal growth factor (Gonnella *et al.*, 1987), milk proteins (Cornell and Padykula, 1969), ferritin and its analogs (Gonnella and Neutra, 1984) by receptor-mediated endocytosis. Other enzyme systems, cytochrome P450 3A4 (CYP3A4) and catalytic activity decrease longitudinally along the small intestine (Paine *et al*, 1997), while P-glycoprotein (P-gp) mRNA levels increase longitudinally increase along the intestine, with lowest levels at the stomach and highest levels in the colon (Fojo *et al.*, 1987).

Metabolism

It is important to understand just how much enzyme activity is present in the GI tract and at which sites. The comprehensive review on the intestinal enzyme systems has been written by Woodley (1994). The greatest threat to therapeutic proteins lies in the lumen of the small intestine, which contains gram quantities of proteases secreted from the pancreas, as well as cellular proteases from the mucosal cells, which are constantly sloughed off from the villie. The second major enzymatic barrier is the brush border membrane of the epithelial cells, which contains at least 15 peptidases that together have a broad specificity and can degrade both proteins and peptides. Woodly (1992) suggested that an orally administered protein might encounter more than 40 different enzymes during passage through the small intestine. This is a qualitative assessment difficult to translate into quantitative information. Furthermore, lysosomal peptidases, estimated at over 60 in number, will also present a barrier to any peptides or proteins endocytosed by the epithelial cells. Considering the 30 amino acid B chain of insulin and just two of the lysosomal peptidases, capthepsins D and B, then exposure of the peptide to just these enzymes might lead to it being split at 20 sites out of a total of 29 (Vaes, 1973).

Interestingly, Garrido *et al.* (1981) have found that the hydrolytic activity for all enzymes (including the subcellular fractions of the aminopeptidase activities) and absorption rates from both the free amino acid and peptide solutions were reduced in bypassed jejunal segments. When large peptides and protein drugs administered to the ileum, there was significant increase in absorption as compared to those drugs administered orally by gavage (Su *et al.*, 2002; Lee and Lee, 2002) supporting the possibility for heterogeneous distribution of the corresponding enzymes in the intestine. The colon has received some attention as a possible site for protein delivery, however, evidence shows that the lumen of the colon contains substantial amounts of peptidase activity, largely because of enzyme production by microorganisms. Therefore, intestinal metabolism act as a concerted barrier to drug absorption. From knowledge of the enzymatic barrier, the strategies for oral protein delivery of enzyme inhibition and the synthesis of enzyme-resistant protein analogues are logical developments. The later approach seems to be more realistic approach for protein drug oral delivery.

In addition, CYP3A4, the major phase I drug metabolizing enzyme in humans, and the MFR1 gene product P-gp are to be considered in designing the delivery system, which are present at high concentration in villus tip enterocytes of the small intestine and share a significant overlap in substrate specificity (Wacher *et al.*, 1998 & 2001; Schellens *et al.*, 2000). Cyclosporin, saquinavir, and a new cystein protease inhibitor of K02 (Morpholine-Urea-Phe-Hphe-Vinyl Sulfone; Axys Pharmaceutical) were metabolized by CYP3A4 and P-gp system (Wacher *et al.*, 1998). A certain drug (Devane, 1997) has prominent affinity for the CYP12 isozyme, lesser affinity for the CYP3A4 and CYP2C isozymes, and minimal affinity for CYP2D6. This profile suggests the need for careful dosage adjustment when used together with some drugs that have a narrow therapeutic range in order to minimize inhibiting their metabolism. Once survived the intestinal enzymatic barriers, protein drugs have to face another presystemic metabolism, blood and hepatic extraction although the transit time is shorter and the enzyme capacity could be smaller than those of the gut (Lehr, 1994).

Permeability

The generally poor permeability of peptide and protein drugs across biological membranes can attributed to their hydrophilic structure and large molecular size (Fasano, 1998c; Fix, 1996; Watts and Fasano, 2000). The mucosal cell permeability determines the rate of entry into the mucosa across the brush-border membrane. The permeation process can be carrier-mediated and paracellular or transcellular. Although no quantitative cross-correlations are established between human and animal permeability, tissue culture, e.g., Caco-2 cell, permeabilities and physicochemical properties such as partition coefficient, advancement in research in these areas is exploitive by many investigators indicating the availability of informative database in near future. Poor membrane permeability of hydrophilic proteins might be overcome by structurally modifying the compounds, thus increasing their membrane partition characteristics and/or their affinity to carrier proteins (Lipka, 1996). A variety of permeability enhancers (Fix, 1996) for peptides, including salicylates, mixed bile salt-fatty acid micelles, chelators, fatty acids, acylcarnitines, surfactants, and medium chain glycerides have been shown in cellular and animal models to increase the absorption of the peptides. These enhancers are not

warranted to work for protein drugs, however, it can be helpful to find a starting point for protein drug delivery.

Transporters

Rapid progress has been made recently in studies on the molecular basis of the intestinal peptide transport system. Intestinal transporters are located in the brush border membrane as well as basolaterial membrane. Each transporter exhibits its own substrate specificity, and some have broader specificities than others (Lee, 2000; Suzuki and Sugiyama, 2000; Tsuji and Tamai, 1996; Yang *et al*., 1999). In addition, the distribution and characteristics of the intestinal transporters exhibit re-gional differences along the intestine, implying diverse physiological functions and in some cases pathological responses. There have been several studies on the mechanism and substrate structure-affinity relationship for this transport system (Arimori and Nakano, 1998; Fricker and Drewe, 1996; Fromm, 2000; Suzuki and Sugiyama, 2000; Wacher *et al*, 1998).

The cloning, expression, and characterization of individual peptide transporters will give additional insight into the rational design of drug molecules as well as delivery system especially for prodrug approaches (Oh *et al*., 1999; Wacher *et al*, 2001). Functional expression of the multidrug resistance gene product, P-gp, as a primary active transporter in the intestinal brush-border membrane leads to net secretion of some drugs such as anticancer agents in the blood-to-luminal direction, serving as a secretory detoxification and as a part of the absorption barrier in the intestine. Grapefruit juice enhances bioavailability of various drugs by inhibiting oxidative metabolism and intestinal secretion P-gp (Spahn-Langguth and Langguth, 2001). Therefore, an understanding of the molecular and functional characteristics of the intestinal membrane transporters will be helpful in the utilization of these transporters for the enhanced oral delivery of poorly absorbed drugs.

Low solubility

For certain proteins with low solubility such as human growth hormone (18 mg/ml), poor absorption was believed to be a major cause for the low solubility. In an attempt to improve the solubility-limited bioavailability associated with these types of compounds, formulators have turned to the use of lipid excipients in which the compounds can be solubilized prior to oral administration. For selection of lipid-based delivery, Biopharmaceutical Classification System (BCS) can serve as a useful preliminary guide (Wasan, 2001). Further elucidation of the physiological aspects of lipid excipients, for instance, inhibition of gut wall efflux mediated by multidrug resistant (MDR) P-gp and presystemic metabolism by CYP3A4 bound to the gut wall, can be expected to define further candidate selection based on the BCS (Wasan, 2001; Wacher *et al*., 1998 & 2001). Nanocapsules and nanoparticulates could help protein molecules to bypass the solubility-limited thus penetration and proteolytic barriers by taken up to the epithelial mucosa via the paracellular pathway (Kompella and Lee, 2001).

Molecular size

In particular peptides and proteins displaying a molecular size greater than 30 Å, the intestinal membrane becomes an important rate limiting factor for drug absorption (Lee, 1991; Bernkop-Schnürch, 2000). Size limitation for protein drug oral absorption could be modulated by a formulation type and an absorption site. The particle sizes employed for current protein oral delivery can be classified into two; 10~1000 nm of nanoparticle and 1~1000 ìm of microparticulates (Fassano, 1998a). The uptake of particles within the intestine increases with decreasing particle size and increasing hydrophobicity (Krueter, 1991). Furthermore, this size dependency in absorption could be site dependent. One study employed a series of polyethylene glycols (PEGs) suggests that compound with a molecular weight up to 4000 might be significantly absorbed (Lipka 1996), assuming appropriate partition behavior and stability. However, several reports on insulin, hGH. EPO, IFN and many other proteins with a molecular weight ranging from 6000 to 30 KDa, showed absorption when administered orally indicating site dependent absorption (Leone-Bay *et al*., 2000). These findings may need further clarification.

Chemical stability

Physical properties of proteins often present significant formulation problems not encountered with conventional organic drug molecules (Fix, 1996), e.g., self-aggregation. The tendency of insulin to form hexamers is well known and the absorption of hexamers is different than that of the monomer. Human calcitonin is also known for self-organization into fibrillar structures with reduced biological activity (Merkle, 1994).

Drug interaction

Drug interactions can occur at any step from absorption to elimination of a drug, and can induce adverse as well as beneficial effects (Gregg, 1999). The main potential for drug interaction is with drugs or compounds that are metabolized by or affect CYP3A4, including imidazoles, grapefruit juice, erythromycin, mibefradil and others (Miller and Spencer, 1998). The pharmacodynamic (PD) consequences may or may not closely follow phar-macokinetic (PK) changes.

Gender difference

Gender-specific PK/PD are for the cases with the differences persisted after normalization for weight. Possible explanations are differences in body composition between men and women and/or physiological changes during the menstrual cycle as well as differences in plasma protein binding secondary to hormonal characteristics (Beierle *et al.*, 1999). In addition, sex differences in drug metabolism may be involved in the higher incidence of adverse reactions in women compared with men (Harris *et al.*, 1995). However, it has not been fully elucidated in the literature whether there is any quantitative and or qualitative difference in proteolytic enzymes and physiology of the absorptive cells in the intestine, which may produce a significant impact on protein drug oral absorption. Sex related differences (Thurmann and Hompesch, 1998) could be shown for Phase I (cytochrome P450) as well as phase II (especially glucuronidation) reactions. Frequent and sometimes clinically relevant gender differences could be identified for drug elimination processes and were predominantly linked to the sex-specific expression of metabolic enzyme systems, e.g., CYP3A4 and CYP1A2. With regard to PD, gender differences have been observed in baseline characteristics as well as in drug response, which might both, at least in part, be the consequence of modulation by sex hormones. Since most of protein drugs are from endogenous sources, it would be important to understand baseline pharmacodynamics in oral drug delivery. Some of the most striking examples identified were in pain therapy and perception, glucose management and arrhythmia susceptibility. CYP3A4, responsible for the metabolism of over 50% of therapeutic drugs, exhibits higher activity in women than in men (Tanaka, 1999) whereas the activity of many other systems involved in drug metabolism may be higher in men than in women (Harris *et al.*, 1995). Women and men also show different PD effect responses to a variety of drugs. While the clinical significance of these sex differences remains to be determined, it is anticipated to be most important in the administration of drugs that have a narrow therapeutic range.

Others

Particle uptake seems to be greater in older animals (Fasano, 1998a). The exact mechanism of this was not fully explained. The presence of food seems to be another enhancing factor for particle uptake possible by increasing intestinal transit time. Hepatic insufficiency reduce the plasma clearance of a number of drugs eliminated by biotransformation and/or biliary excretion, but it can also affect plasma protein binding which in turn could influence the processes of distribution and elimination (Verbeeck, 1998). In addition, reduced liver blood flow in patients with chronic disease will decrease the systemic clearance of flow limited (high extraction) drugs and portal-systemic shunting may substantially reduce their presystemic elimination (first-pass effect) following oral administration. When selecting a drug and its dosage regimen for a patient with liver disease additional considerations such as altered phar-macodynamics and impaired renal excretion (hepatorenal syndrome) of drugs and metabolites should also be taken into account. Consequently, dosage reduction is ne-cessary for many drugs administered to patients with chronic liver disease such as liver cirrhosis. Protein drug PK are not appreciably affected by age, gender or race when differences in renal function, and body mass and composition are taken into account (Fasano, 1998a & b).

APPROACHES FOR ORAL DELIVERY

The strategy employing enzyme inhibitor(s) and absorption enhancers to protect from the various enzymes and to help to overcome its own hurdles, i.e., hydrophilicity, molecular size, charge, and etc., seems to be the most popular approach for protein oral delivery (Lehr, 1994). Second realistic approach could be modifying protein structures to more stable analogs by chemical synthesis. Targeting the specific absorption site and dosage form modification, i.e., lipid vesicles, colloidal carrier systems with and without mucoadhesive polymers could be another approaches to improve protein absorption. A novel and perhaps less complex oral protein drug technology could be based on combination of several approaches that are described as below.

Enzyme inhibiton

Due to the nature of enzyme distribution and quantities, a use of only enzyme inhibitor(s) for protein oral delivery will be extremely inefficient. However, some successful results have been reported for insulin administered with sodium glycocholate, camostat mesilate and bacitracin to rats by Yamamato *et al.* (1994) and with FK-448 and other protease inhibitors by other investigators (Fuiji *et al.*, 1985; Lee, 1991). CYP3A4 inhibitor showed marked increase in the oral bioavailability of cyclosporin (Foradori *et al.*, 1998). Utilization of both inhibitor cocktails and a specific absorption site with less enzyme distribution showed improvement in the absorption of large peptides, cholecystokinin and enkephalin analogs (Su *et al.*, 2002; Lee and Amidon, 2002) and protein drugs (Lee and Lee, 2002).

A new and interesting possibility for protein drug oral delivery makes use of a polymer and enzyme inhibitor conjugate system (Bernkop-Schnurch *et al.*, 1997a & 1997b) in which the embedded therapeutic agent is protected from enzymatic degradation. Chitosan and its

derivatives showed multiple effects of enhancer, enzyme inhibitor and mucoadhesion to improve the absorption of insulin, calcitonin, and buserelin following oral administration (Bernkop-Schnurch, 2000). In similar work (Kratzel *et al.*, 1998) the enzyme inhibitor, pepstatin analogs were covalently joined to mucoadhesive polymers so that the inhibitor molecules were freely accessible to the enzyme as a substrate. This system provided advantages toward increasing contact time with the GI mucosa and maintained a controlled and sustained drug release from the adhered polymer and reducing unwanted toxic effects of the inhibitor because of its attachment to the no-nabsorbable polymer backbone. However, from a practical point of view, the utility of this approach may be limited by high manufacturing cost (Leone-Bay *et al.*, 2000).

Carrier or enhancers

Avoiding temporary to long-lasting membrane damage, carrier molecules or enhancers improving the epithelial cell permeability of protein drugs were synthesized by Leone-Bay *et al* (1996 & 2000), which were low molecular weight, peptide-like compounds that interact no covalently with the protein drug. This approach was successful for hGH oral absorption with a mean peak concentration of 55 ng/mL in primates (Leone-Bay *et al.*, 1996). Another group's research employing the delivery agent resulted in the bioavailability increase of 50% to 10^4 % for hGH, interferon alpha-2b, and insulin (Stoll *et al*, 2000). Permeability increases in the presence of carrier molecules for hGH, IFN, and insulin were 0, 350, and 800%, respectively, indicating proportional increase of bioavailability to permeability (Stoll *et al*, 2000). The ability to increase oral absorption of G-CSF and EPO by covalent coupling to vitamin B_{12} has been investigated (Russlel-Jones *et al.*, 1999; Russlel-Jones, 1998). This absorption occurs via receptor-mediated endocytosis and an essential requirement is the presence of the intrinsic factor of a mucoprotein. The mucoprotein complex reaches the ileum, where resorption is mediated by specific receptors. Possible disadvantages of the vitamin B_{12} mediated delivery are the limited capacity of this active transport mechanism and the interference with vitamin B_{12} absorption in chronic therapy (Jung *et al*, 2000).

Prodrugs and analogs

Altering physicochemical properties of protein drugs seems to be the most easiest approach (Samanen *et al*, 1996; Woodley, 1994) warranting unaltered or intensified efficacy although it requires the synthesis of a new chemical entity (Leone-Bay *et al.*, 2000). Changes can be made in liphophilicity, charge, molecular size, solubility, configuration, isoelectric point, chemical stability, enzyme liability, and affinity to carriers to enhance the absorption and systemic circulation. Two specific approaches made for peptide oral deliver by Samanen *et al.* (1996); the chemical modification of peptide amide bond to enhance intestinal permeability and the design of compounds bearing nonpeptide templates, which were more amenable to the discover of compounds with oral activity, from peptide pharmacophore models, e.g., SB 208651 designed from the peptides SK&F 106760 and SK&F 107260.

Optimal absorption site

Kompella and Lee (2001) suggested that identification of the optimal absorption site for a given peptide or protein is the first step towards the design of a delivery system that maximizes absorption. Regional variations in the penetration barriers to peptides may result in regional differences in their absorption. For instance, M-cells located on the dome epithelium of gut-associated lymphoid tissue are known to be capable of sampling macromolecular antigens from the ileum through an endocytotic pathway (Keljo and Hamilton, 1983). Controlling the absorption site to deliver proteins and large peptides drugs has been attempted with some success in improving oral bioavailability (Lee and Amidon 2002; Lee and Lee, 2002; Su *et al.*, 2002). Researchers searched for absorption sites with less enzyme distribution and avoiding hepatic the first-pass effect and found, thus far, the oral cavity (buccal) and colon as alternative sites for protein oral delivery.

Buccal delivery

The oral mucosa has greater permeability and perfusion than the skin while the oral cavity provides an environment almost free from the acidity and protease activity encountered elsewhere in the gastrointestinal tract (Senel *et al*, 2001). In addition, blood vessels of the oral mucosa drain directly into the jugular vein avoiding first-pass extraction by the liver. Buccal delivery permits easy removal in the event of adverse reactions. Recent study revealed the presence of aminopetidase activity on the buccal mucosa and its inhibition by enzyme inhibitors (Walker *et al.*, 2002). Complete review on the regional variation in oral mucosal drug permeability written by Kurosaki and Kimura (2000) is available. In this report, the structure and composition of the mucosa at different sites in the GI tract, factors affecting mucosal permeability, and formulation factors including penetration enhancement relevant to the design of systems targeting oral mucosal delivery were reviewed. Many oral mucosal delivery systems for numerous peptide/protein drugs have been described since the first attempt at a bioadhesive system by Nagai in the early 80s (Nagai, 1988), yet none have been reached the market. Transforming growth factor beta-3 (TGF-â3) has shown that it is temporarily arrest the

oral mucosal basal cell proliferation (Spijkervet and Sonis, 1998). Delivery of calcitonin, LHRH, and glucagon-like-peptide I as an adhesive tablet showed the bioavailability of 37% (Alur *et al*, 1999), 100% (Nakane *et al*., 1996), and 41% (Gutniak *et al*, 1996), respectively. Exciting challenges remain to influence the bioavailability of drugs across the oral mucosa. Nonetheless, there are still many issues to resolve before this effective and convenient route of drug delivery can be thoroughly and safely utilized (Senel *et al*, 2001).

Colon delivery

A common belief is that colonic delivery for orally administered protein drugs is possible because of the postulated low proteolysis activity in the large intestine, an assumption that requires further verification (Rubinstein, 1995). Yet, other opportunities for colonic delivery of drugs also exist. Some recent examples include bypassing small intestine metabolism, achieving constant absorption rates for some molecules, and delivering cationized antioxidant enzymes to the colonic epithelium. Colonic delivery of insulin after coating with azo-cross-linked copolymer of styrene and HEMA aiming for activation by microflora of large intestine and intestinal delivery of enterocoated cyclosporin, are examples of site-specific delivery cases (Kompella and Lee, 2001; Saffran, 1986). Baluom *et al*. (1997) also achieved about 15% of bioavailability after colonic administration of salmon calcitonin using submicron emulsions containing Carbopol® 940. Another problem with protein delivery to the colon is various transit time ranging from a few hours to ten hrs (Macfarlane *et al*., 1989).

Targeting transporter

Recent advances in gene cloning and molecular biology techniques make it possible to study the characteristics and distribution of transporters at the molecular level. Based on molecular characterizations of membrane transporters and accumulated biochemical data on their specificities and kinetics, structural modification and targeting of a specific transporter is a promising strategy for the design of drugs that improve bioavailability and tissue distribution (Wacher *et al*., 2000; Tsuji and Tamai, 1996). The peptide transporter is limited to peptides of less than 4 amino acids (Russell-Jones, 1998). The bile acid transporter is limited to peptides of less than 400 MW, while the vitamin B_{12} transporter is limited to pharmaceuticals that require less than 40 fmoles per day.

Colloidal carrier systems

Various colloidal systems have been studied for absorption enhancement of proteins, such as sub-micron emulsions, lipid suspensions, liposome, polymeric nano- and microparticles. Controversy still exists on physicochemical factors governing gastrointestinal uptake, including size, size distribution, consistency, hydrophobicity and surface properties (Jung *et al*., 2000). Prolonged contact of nanoparticles (NP) with absorptive cells may be achieved using bioadhesive materials. Bioadhesion could be followed by NP uptake in a second step. Therefore, biomaterials with both adhesive and protective properties would be desirable for oral protein delivery in order to assure the drug stability and thus improved bioavailability (Sakuma *et al*., 2001).

Nanoparticles

Nanoparticles (NP), defined as solid particles with a size in the range of 10-1000 nm, allow encapsulation of the protein drugs inside a matrix, protecting them against enzymatic and hydrolytic degradation. Various biomaterials of polymers, lipids, lectins and etc. could be employed to make NP using techniques of emulsion polymerization, interfacial polymerization, emulsification evaporation, solvent displacement, salting out, emulsification diffusion, and desolvation (Allemann *et al*., 1993; Quintanar-Guerrero *et al*., 1998). The solvent displacement and salting out have received increasing importance, because they provide less stress to protein drugs. The physicochemical properties of NP and their behavior on exposure to physiological media are greatly dominated by their chemical structures and surface characteristics (Jung *et al*., 2000). Mucus layer and the Peyers patches (Jani *et al*, 1989) were considered to be uptake site for NP.

Bio(muco)adhesion

Mucoadhesive oral drug-delivery systems have received considerable attention over the last decade. Utilizing bioadhesive polymers to prolong and/or to intensify the contact between controlled-release dosage forms and the stomach or gut mucosa has also shed new light on the potential of mucoadhesive polymers. In a series of cationic, anionic, and neutral polymers, anionic poly (acrylic acid) (PAA) was found by Park and Robinson (1984) to show the highest binding affinity to human conjunctival epithelial cells. PAA could be identified as a potent inhibitor of proteolytic enzymes as well. There is also increasing evidence that the interaction between various types of bio(muco)adhesive polymers and epithelial cells has direct influence on the permeability of mucosal epithelia. It was demonstrated that the adhesion or binding of proteins to the intestinal epithelial cell surface lead to enhanced uptake by nonspecific receptor-mediated endocytosis (Carreno-Gomez *et al*., 1999). One area to improve in this technology is to increase the localization or retention in any desired regions (Kompella and Lee, 2001). The inability of mucoadhesive systems to

remain for longer periods at the site of attachment is partly due to the high turnover and sloughing rates of the mucus to which the system attached (40~270 min in rats, Lehr *et al.*, 1990) and partly due to the possible displacement of the delivery system from binding to the mucus layer by soluble mucus in the luminal contents (Allen *et al.*, 1984). Rather than being just adhesives, mucoadhesive polymers may therefore be considered as a novel class of multifunctional macromolecules with a number of desirable properties for their use as biologically active drug delivery adjuvant (Lehr, 1994).

Liposomes

Liposomes have been abandoned due to insufficient stability under in vivo condition (Kreuter, 1991). In an application to vaccines, homogeneous unilamellar liposomes of ~100 nm were reported to be taken up into M cells (DiBiase and Morrel, 1997). However, studies on the vaccine liposome led to the overall conclusion that liposomes are ineffective as adjuvant for the nonparticulate, naturally weak immunogens used in the investigation. A mucoadhesive liposomal system prepared by coating a negatively (phosphatidyl choline) or positively charged (salicylic acid) liposome suspensions with mucoadhesive polymer solutions, such as chitosan and carbopol showed some success in significant absorption of protein drugs, e.g., insulin and calicitonin (Takeuchi *et al.*, 2001). Recently, the liposome technology has been revisited (Lasic and Papahadjopoulos, 1995) and evaluated. Various cytokins in liposomes (Anderson *et al.*, 1994) and a multivesicular liposomes characterized by their unique structure of multiple non-concentric aqueous chambers surrounded by a network lipid (Ye *et al.*, 2000) have been tested and obtained satisfied pharmacodynamic effects of model protein drugs.

Microencapsulation

Poly(lactide/gycolic acid) (PLGA) polyester microspheres have been used for long-acting injectable sustained release formulations of protein drugs (Lee *et al.*, 1997) due to its biodegradable and biocompatible property. This PLGA microspheres have also been investigated as potential antigen delivery systems for oral vaccines of staphylococcal enterotoxin B (SEB) (Eldridge *et al.*, 1990). Viruses were also successfully encapsulated in PLGA microspheres for oral delivery (Ray *et al.*, 1993). Zein, one of the typical prolamines and a major storage protein of corn (Larkins *et al.*, 1984), is insoluble in aqueous media yet is degraded over time by protease enzymes to constituent peptides and amino acids showing mucoadhesive properties. Alkermes, Inc. (Cambridge, MA) was successful to encapsulate calcitonin, EPO, sesmopressin, vasopressin, and insulin into the zein microsphere system for oral administration (DiBiase and Morrel, 1997).

Emulsions

Using this technology the method of transport in the gastrointestinal mucosa was shown to be transcellular by enhancing membrane permeability with oil in the formulation (Torres-Lugo *et al.*, 2000). Calcitonin and hGH in microemulsion for an oral formulation had been developed and tested in humans for commercialization by Cortecs Int. Ltd. (Middlesex, U.K.) and Affinity Biotech, Inc (Aston, PA) (New *et al.*, 1994; DiBiase and Morrel, 1997).

SPECIFIC EXAMPLES

Up to now, the formulation development can be administered orally that is clinically approved has been in limited success. US FDA approved oral formulations that is currently in use are listed below for case discussion.

Vaccines

For vaccination, enteric delivery may result in the induction of a mucosal immune response against pathogens, which colonise and invade the mucosa (Thomas *et al.*, 1996). The problem for oral vaccine delivery is the poor immune response by stimulating mucosal lymphoid inductive sites as intestinal Peyer□s patches results in parallel immune responses manifested by the appearance of S-IgA antibodies in the external secretions of remote glands (Mestecky *et al*, 1997). Vaccines require for the induction of humoral immune responses (Mannino and Gould-Fogerite, 1995). Currently FDA approved oral vaccine formulations are enteric coated adenovirus tablet, typhoid fever bacteria in gelatin capsule, polio virus in liquid, and lyophilized rotavirus (Burke *et al.*, 1999). The successful use of live attenuated viral and bacterial vaccines depends not only on the proper choice and delivery of the microorganisms, but also on maintaining the sufficient potency required for an immune response (Babiuk *et al.*, 2000). The inherent labiality of live organisms presents a particular formulation challenge in terms of stabilizing and preserving vaccine viability during manufacturing, storage, and administration (Burke *et al.*, 1999).

Neooral®(cyclosporin)

Neoral is an improved formulation of cyclosporine through microemulsion technology, which could generate more reproducible serum profiles and reduce nephrotoxicity (Corbett and Ross, 1998). A dose reduction of 30% yields pharmacoeconomic savings as well. Combined use with CYP3A inhibitors, ketoconazole or diltiazem altered the PK of cyclosporin resulting in further

improvement of 3~5 fold oral bioavailability in humans (Foradori *et al.*, 1998).

FUTURE TECHNOLOGY

A few new technologies or concepts, which have not been or actively tested for oral delivery for various reasons, are discussed in this section. In addition, necessary assisting technologies to enhance this field are also described here.

Vitamin B_{12} conjugates

Vitamin B_{12} conjugates to deliver protein drugs by the oral route have been pursued by a few investigators (Russlel-Jones *et al.*, 1999; Russlel-Jones, 1998). This system relies upon the natural uptake mechanism for vitamin B_{12} to cotransport peptides and proteins linked to the vitamin B_{12} from the intestine to the circulation. In an exciting extension to the technology, it has been found that it is also possible to transport nanoparticles, linked to the vitamin B_{12}, into the circulation. Such nanoparticles can potentially be loaded with peptides or proteins of choice, and so protect theses molecules from degradation in the intestine, while simultaneously transporting them into the circulation. This uptake system could help for protein drug to overcome both metabolism and absorption problems (Russel-Jones, 1998). Habberfield *et al.* (1996) showed the possibility to utilize this transport system to promote the intestinal uptake of G-CSF and EPO. These findings are an important step in realizing the possibility of delivering almost all peptides and proteins via the oral route.

Transport vector

Utilizing transport vectors to enhance the absorption of impermeable peptide drugs through the blood-brain barrier (BBB) have been attempted and the extensive work reviewed by Bickel *et al.* (2001). Although the anatomy and physiology of the intestinal membrane is quite different from those of the BBB, the transcellular delivery approach could be applicable to protein drug delivery. Chimeric peptides were formed when non-transportable peptide therapeutics are coupled to a BBB drug transport vector. Transport vectors are proteins such as cationized albumin, or the OX26 monoclonal antibody to transferring receptor; these proteins undergo absorptive-mediated and receptor-mediated transcytosis through the BBB (Bickel *et al.*, 2001). Finding the transport vectors or cell penetrating proteins would not be difficult for intestinal membranes.

Potent inhibitors/enhancers

Ideal protease inhibitors are effective at preventing or suppressing enzymatic degradation in the intestine yet safe. Bowman-birk inhibitor (BBI) is a protein of a molecular weight of 8,000 with a well-characterized ability to inhibit trypsin and chymotrypsin (Bell and Ovalle, 2001; Kennedy, 1998). BBI has been extensively studied, both as purified and as an extract of soybeans. Enriched in BBI called BBI concentrate (BBIC). The usage of BBIC for possible (ideal) enzyme inhibitors with other helpful technologies to deliver protein drugs orally is under investigation (Lee and Lee, 2002). By knowing the fact that the biggest hurdle for protein oral absorption is the metabolism, it is urged to develop a universal yet biocompatible enzyme inhibitor that can enhance the protein absorption without causing damage to intestinal membranes. Developing reversal agents for P-gp, which can substantially increase oral absorption (Spahn-Langguth and Langguth, 2001), could be an alternate approach to aid the inhibitor strategy.

Assay development

The pharmacokinetic analysis of protein drugs involves the understanding of analytical methods capable of detecting these agents in biological fluids and recognition of several factors that may have an impact on the drug concentration-time curves (Piscitelli *et al*, 1997). Enzyme-linked immunosorbent assays (ELISA) have become the most common method of detection and commercial kits are available for a wide variety of protein drugs. Monoclonal antibody products are sensitive, have minimal cross-reactivity and are relatively inexpensive when compared with high performance liquid chromatography (HPLC). However, the primary limitation of these assays is their inability to measure biologically active protein. Conversely, bioassays do measure a biological event (i.e. proliferation or cytotoxicity) but are generally not used because of their high cost, long assay completion time, lack of specificity, poor sensitivity and influence of environmental conditions on the outcome. Inhibition by many other drugs has been demonstrated in several in vitro systems and animal models, although clinical data are currently limited. An increased understanding of the many factors that can alter the analysis and pharmacokinetics of protein drugs and development simple yet sensitive and specific analysis methods are essential for protein drug oral delivery.

CONCLUDING REMARKS

The GI enzymatic barrier for protein oral delivery is formidable. Overcoming this barrier is one of the technical challenges for pharmaceutical scientist. Other obstacles associated with protein oral delivery remain challenging as well. However, significant progress has been made with each of the obstacles and with certain proteins. The

development of the composite formulation, which improves bioavailability yet meets regulatory requirements for reproducibility and intra-and inter-subject variability, and reasonable manufacturing costs, will be hard but the future of this infant field is bright.

ACKNOWLEDGEMENTS

The author thanks Prof. Myung G. Lee for his support and provision of all possible means to prepare this paper, and Mr. Richard Chae for his excellent technical assistance. The authors thanks also go to Prof. Chang-Koo Shim, the editor-in-chief of APS, for his invitation and encouragement to write this review article.

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