EPIDERMAL GROWTH FACTOR RECEPTOR IN EQUINE

GASTRIC STRATIFIED SQUAMOUS MUCOSA:

EFFECT OF PROGRESSIVE ULCERATION ON RECEPTOR DENSITY

by

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(ABSTRACT)

The objective of the study reported here was to document the distribution of epidermal growth factor receptor (EGFr) and quantitate receptor density in normal as well as ulcerated equine gastric squamous mucosa. Fifteen horses with endoscopically normal stomachs were divided into three equal groups. Group 1 was a normal control. A protocol that alternated 24 hour periods of free-choice hay with 24 hours of feed deprivation was utilized to induce squamous mucosal gastric ulceration in Group 2 (48 hours total off-feed) and Group 3 (96 hours total off-feed). Gastric tissue was collected from 3 stomach locations at post-mortem examination and an avidin-biotin immunoperoxidase technique was developed to stain the formalin-fixed tissue for EGFr. A computerized image analysis system was used to measure EGFr area and mean intensity values at four sites within the epithelium from the basal cell layers to the lumen in the ulcer/erosion margin, erosion bed, and 10-14 mm distant from the lesion.
Additionally, EGFr area and intensity values were measured in epithelial cells adjacent to capillaries.

Results showed that mean EGFr area and intensity were greatest in the basal layer, and both progressively diminished toward the lumen. EGFr areas in the ulcer/erosion margin and distant from the lesion margin in Groups 2 and 3 were significantly greater than comparable sites in the normal epithelium of Group 1 (p<0.05). Tissues from Group 3 had significantly greater EGFr area in the margin than Group 2 (p=0.01). EGFr area in the erosion bed of Group 2 was significantly less than normal tissue from Group 1 (p=0.027), whereas Group 3 EGFr erosion bed area was significantly greater than Group 1 (p=0.042). EGFr intensity in the lesion margin, erosion bed, and distant from the margin in Groups 2 and 3 was significantly less than normal tissue from Group 1 (p<0.05). EGFr area in cells adjacent to epithelial capillaries of Group 3 was significantly greater than that of Group 1 (p<0.05). These results are consistent with an EGFr ligand (EGF or TGF-alpha) being a factor in ulcer healing, and that repair processes begin with the onset of peptic injury to gastric squamous epithelium.
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EPIDERMAL GROWTH FACTOR RECEPTOR IN EQUINE GASTRIC STRATIFIED SQUAMOUS EPITHELIAL MUCOSA: EFFECT OF PROGRESSIVE ULCERATION ON RECEPTOR DENSITY

A. MATERIALS AND METHODS
B. RESULTS
C. DISCUSSION
D. CONCLUSIONS

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VITA
INTRODUCTION

Peptide growth factors are important regulatory substances produced by normal cells. They initiate signals that control cell growth and differentiation, as well as influence a myriad of metabolic processes in the body.¹ Epidermal growth factor has been studied intensively for the last 30 years since its discovery in 1962, although our knowledge of the full spectrum of growth factors and their actions is still in its infancy.

Epidermal growth factor (EGF) has many important physiologic effects with regard to the gastrointestinal tract, including mucosal protection,² inhibition of gastric acid secretion,³ and mucosal trophism.⁴ It has been clearly demonstrated that EGF plays a role in gastric ulcer healing in laboratory animals,⁵ and EGF has also been shown to enhance healing of topical wounds⁶ and corneal ulcers.⁷ Gastric ulceration is a prevalent disorder of horses and foals,⁸,⁹ and the majority of ulcers occur in the stratified squamous epithelial mucosa of the stomach.¹⁰,¹¹ Previous studies regarding observation of the EGF receptor in gastric mucosa have focused on the glandular stomach of laboratory animals such as the rat, mainly as an extrapolation for the human glandular stomach. Significant increases in EGF receptor density have been found in the glandular mucosa during gastric ulcer healing in rats.¹²
leading to the conclusion that EGF plays an important role in ulcer repair. We suspect that growth factors also play a necessary role in the healing of gastric squamous epithelial erosions and ulcers in the horse.

MORPHOLOGY

Gross Anatomy of the Equine Stomach

The stomach receives food from the esophagus and initiates the digestive process. Domestic mammals possess a variety of stomach sizes and shapes, depending on the type of food they consume. The composition of the stomach lining also varies between species. The horse has a simple stomach that consists of one compartment, similar to the pig, dog and cat. It is a composite stomach, being lined with both glandular and non-glandular mucosa.

Food from the esophagus enters the stomach through the cardia, is contained within the body of the stomach, then exits into the duodenum through the pylorus. The stomach is a J-shaped, curved sac with a convex greater curvature and concave lesser curvature extending from the cardia to the pylorus. The fundus of the stomach extends dorsally to a small sac that projects above the level of the cardia. The equine stomach lies in the abdominal cavity mostly to the left of the midline, with the body of the stomach being between the ninth and twelfth intercostal spaces.¹³
The composite stomach of the horse contains glandular and squamous epithelial mucosal surfaces. The glandular tissue appears reddish brown in color, and covers the body of the stomach and the pyloric antrum. The fundus and a small portion of the body of the stomach is composed of squamous epithelial mucosa that is smooth and whitish in color. The junction of the glandular and squamous epithelial mucosal surfaces is an irregular, raised ridge called the margo plicatus.

Gastric Histology
The wall of the stomach consists of an epithelial mucosa, submucosa, muscular layer, and outer serosa. The mucosa in the fundus of the equine stomach is composed of stratified squamous epithelium histologically similar to the esophagus and oral cavity. The squamous epithelium can have varying thicknesses depending upon the location in the fundus, and is made up of multiple cell layers. The stratum basale is the germinal layer, and consists of cuboidal cells that lie just above the basement membrane. Above the stratum basale, the stratum spinosum is composed of polyhedral-shaped cells, transforming from cuboidal to flat cells as they migrate toward the stomach lumen. This layer is noted for spinous processes between contiguous cells. The next layer toward the lumen is the stratum granulosum, consisting of spindle shaped
cells containing keratohyalin granules, which are precursors to keratin. The stratum corneum is located closest to the lumen of the stomach, and is composed of closely packed, flattened dead cells without nuclei.\textsuperscript{14} In the equine stomach, layers of keratin can overlie the cornified cells.

The glandular mucosa of the fundus, stomach body and pyloric antrum is occupied by simple, tubular gastric glands which open into gastric pits. The inner surfaces of the gastric pits are lined with simple, tall columnar epithelial cells. The \textbf{cardiac gland region} consists of highly branched, coiled seromucous glands. In the horse, the cardiac glands are located in a narrow zone along the margo plicatus.\textsuperscript{13}

The \textbf{proper gastric gland region} covers the body of the stomach. The glands are oriented perpendicularly to the surface of the mucosa. They are tightly packed, and consist of a base, body, neck, and upper isthmus. The gastric glands contain primarily three cell types: 1) Chief cells are found in the base and body of the gastric glands. They are pyramid-shaped, basophilic cells (Hematoxylin-Eosin stain) with apical secretory granules containing pepsinogen, the precursor to pepsin. 2) Parietal cells are spheroidal, acidophilic (HE stain), and are found in the neck of the gastric glands. These cells secrete hydrochloric acid into the lumen of the gland.
3) Mucus neck cells line the neck of the gastric glands. They are low columnar in shape, pale-staining (HE stain), and are interspersed between the parietal cells.

The pyloric gland region in the horse includes the pyloric antrum and pyloric canal. Pyloric glands are short, simple or branched tubes similar to the cardiac glands, that open into deep gastric pits. The pyloric glands are composed of mucus producing cells.

The gastric glands in the body and antrum also contain other cells important to gastric function. G cells release gastrin, D cells produce somatostatin, mast cells produce histamine, and enterochromaffin-like (ECL) cells release mediators such as histamine, serotonin, secretin, and vasoactive intestinal peptide (VIP).¹⁴

The lamina propria lies immediately beneath the epithelium. This loose connective tissue contains blood vessels, nerve fibers, and various cells types including fibroblasts, lymphocytes, and macrophages. The muscularis mucosa divides the mucosa from the submucosa, and is composed of an inner circular and outer longitudinal layer. The submucosa is also a loose connective tissue containing vasculature, lymphatics, and ganglionic cells of the submucosal plexus.¹⁵
FIGURE A: Stomach of the horse opened on the visceral surface

a Cardia; b Fundus; c Body; d Pyloric antrum; e Pyloric canal; f Pyloric orifice; g Esophagus; h Duodenum; i Greater curvature; k Lesser curvature

1 Cardiac sphincter; 2 Non-glandular squamous mucosa; 3 Margo plicatus; 4 Region of mixed cardiac and pyloric glands; 5 Region of proper gastric glands; 6 Pyloric gland region; 7 Muscular ring; 8 Pyloric sphincter; 9 Accessory pancreatic duct; 10 Major duodenal papilla

(From Schummer, Nickel, and Sack: The Viscera of the Domestic Animals 1979; p 182)
The muscular layers of the stomach consist of an internal oblique, inner circular, and outer longitudinal layer. The internal oblique fibers are only present on the fundus and body of the stomach.

The serosa is the outer layer of the stomach, and is continuous with the greater omentum at the greater curvature, the lesser omentum at the lesser curvature, and the gastrophrenic ligament.

**Gastric Blood Supply and Nervous Innervation**

The left gastric artery supplies blood to the stomach, as do branches of the celiac artery, the splenic artery and the hepatic artery. The gastric veins return blood from the stomach to the portal vein. Parasympathetic innervation of the stomach is through the vagus nerve. Long preganglionic fibers travel to the stomach wall, where they synapse on neurons of the submucosal plexus and the myenteric plexus, which are located between the circular and longitudinal muscle layers. Short postganglionic fibers synapse with the myenteric and submucosal plexuses, then travel to the mucosa. Sympathetic control is exerted by nerve fibers which pass through the celiac ganglion to the stomach. Postganglionic sympathetic fibers synapse with neurons of the myenteric and submucosal plexuses.\(^{13,16}\)
GASTRIC PHYSIOLOGY

Hydrochloric Acid Secretion

The stomach stores food and participates in the digestive process. One of the primary functions of the stomach is to secrete hydrochloric acid, which breaks down connective tissue and muscle fibers,\textsuperscript{17} and kills bacteria in the stomach.\textsuperscript{17,18} Acid activates pepsinogens and provides a low pH environment for pepsin action,\textsuperscript{17} although gastric acid secretion is not required for adequate absorption of nutrients to occur.\textsuperscript{19}

Parietal cells produce hydrochloric acid by activation of the gastric proton pump, an apical membrane-bound hydrogen-potassium-ATPase.\textsuperscript{20} Activation of the proton pump requires an increased concentration of the intracellular messengers cyclic AMP and cytosolic free calcium.\textsuperscript{21} When the parietal cell is stimulated, the previously inactive proton pump is translocated to the apical or canalicular membrane, and increased ATP production provides energy for the hydrogen-potassium ATPase enzyme to transport hydrogen from the cell into the canalicular space in equal exchange for potassium. As hydrogen ions are secreted, a cotransport pathway is activated at the apical membrane to secrete chloride and potassium back into the lumen, resulting in net hydrochloric acid secretion.\textsuperscript{19,21} To maintain a neutral intracellular pH, bicarbonate is transported out of the parietal cell across the
basolateral membrane in exchange for chloride.\textsuperscript{22}

Basal and stimulated gastric acid secretion are variable among different species.\textsuperscript{23,24} Continuous basal acid secretion has been found in the human, monkey, pig, chicken, rodent, and horse, in contrast to the dog and cat, in which basal acid secretion is minimal.\textsuperscript{24} In humans, basal gastric acid secretion demonstrates a circadian rhythm, with highest basal acid output and gastric acidity occurring in the late evening, and lowest in the morning hours.\textsuperscript{25} Mechanisms for the rhythmicity are not fully understood. The horse has a continuously variable gastric acid secretion that occurs without the ingestion of feed.\textsuperscript{26} Gastric juice volume, pH, hydrogen ion concentration, and acid output are variable under basal conditions.\textsuperscript{27} In contrast to the human, no circadian rhythm of gastric acidity has been detected in the horse.\textsuperscript{28} Both the horse and human demonstrate variation in basal output from one day to the next, as well as from one individual to another.\textsuperscript{24} The volume of gastric secretions in the horse under basal and pentagastrin stimulation are much greater than that of the human and dog on a kilogram body weight basis (ml/kg/hr).\textsuperscript{24} This large volume of secretion may be related to the continuous feeding habits of the herbivore, in which prolonged stimulation of gastric secretions is necessary for digestion.\textsuperscript{24} Basal acid output in the horse on a kilogram body weight basis
is greater than in the human, but after stimulation by pentagastrin, maximal acid output is similar. Maximal acid output in the horse implies production by approximately 4.6 billion parietal cells in the stomach.

Equine gastric acidity studies have shown that because of continuous basal secretion, gastric pH in the horse remains highly acidic when feed is withheld. Median 24-hour gastric pH in unfed horses was significantly lower (1.55) than horses fed free choice hay, (3.1). Unfed horses also maintained a gastric pH below 2.0 for a significantly greater percentage of the 24 hour period (76%) compared to 30% for horses that were fed free choice hay.

The equine stomach demonstrates a dorsal to ventral pH gradient, with the highest pH being at the mucosal surface of the squamous fundus (5.46±1.82). The pH of the squamous fundus of the stomach in the adult horse is significantly greater than that adjacent to the margo plicatus (4.12±1.62) and the glandular fundic mucosa (3.09±1.90).

Regulation of acid secretion
There are three primary pathways that influence hydrochloric acid secretion; neurotransmitters are released from postganglionic nerve fibers in the stomach wall (neurocrine),
hormones are carried to parietal receptors via the bloodstream (endocrine), and tissue factors are released locally which diffuse across intercellular spaces (paracrine). Acetylcholine, gastrin, and histamine are three direct stimulants to gastric acid secretion via the neurocrine, endocrine, and paracrine pathways respectively. Three major receptors have been identified on the human parietal cell, and include muscarinic, gastrin, and histamine type 2.

The central nervous system provides neurocrine control of gastric acid secretion by vagus nerve fibers that innervate parietal cells in the stomach antrum. The neurotransmitter acetylcholine directly stimulates the parietal cell by increasing cytosolic calcium levels, which activates the proton pump. Acetylcholine also reduces the release of somatostatin, an inhibitor of gastric acid secretion. Nerve signals can originate in the brain, or secretory signals can be generated by local reflexes within the enteric nervous system. In addition to stimulating acid production, acetylcholine also induces secretion of pepsinogen by chief cells and mucus by mucus cells.

Vagal stimulation induces the release of gastrin from G cells in the antrum and fundus, which is absorbed into the systemic circulation as a hormone, then transported in the bloodstream
back to receptors on the basolateral surface of the parietal cells.\textsuperscript{16,19} Gastrin increases parietal intracellular calcium levels, which results in translocation and activation of the proton pump.\textsuperscript{21} Gastrin release is also stimulated by gastric distention, an increase in intraluminal pH, and protein breakdown products such peptic hydrolysates.\textsuperscript{18}

Paracrine regulation of gastric acid secretion is exerted by glandular mucosal cells that secrete histamine, somatostatin, and serotonin.\textsuperscript{18} Histamine is thought to be the major stimulus to acid production by the parietal cell,\textsuperscript{21,31} and is released by mast cells and enterochromaffin-like (ECL) cells.\textsuperscript{32} Histamine binds to the parietal cell H\textsubscript{2} receptor and activates adenylate cyclase, resulting in increased cyclic AMP and hydrochloric acid secretion.\textsuperscript{21} In mammals, enterochromaffin-like cells are located primarily in the basal third of the gastric glands, and mast cells are uniformly distributed throughout the thickness of the mucosa.\textsuperscript{32} Because the parietal cells are located in the middle third of the gastric glands, there is speculation that histamine reaches the parietal cell receptors via capillary transport from the glandular base\textsuperscript{32} and diffusion through interstitial spaces.\textsuperscript{19,32}

Acetylcholine and gastrin stimulate the release of histamine from paracrine cells close to the parietal cell, further
enhancing gastric acid secretion. Histamine appears to be a necessary cofactor in the stimulation of the parietal cell, since H₂ receptor antagonists inhibit the acid secretion-response to vagal stimulation, cholinergic agents, and gastrin, as well as histamine-stimulated acid secretion. It has been suggested that histamine's induction of the cyclic AMP pathway may be involved in the activation of the increased intracellular calcium pathway stimulated by acetylcholine and gastrin.

**Inhibition of acid secretion**

Somatostatin is a potent inhibitor of gastric acid secretion. D cells in the fundus and antrum produce somatostatin, and its effect on gastric acid secretion is exerted by endocrine and paracrine pathways. Intravenous infusion of somatostatin causes partial inhibition of gastric acid secretion, and paracrine control is suggested by the close proximity of D cells to parietal cells within the gastric glands. In some species, cytoplasmic processes from somatostatin-producing D cells extend to parietal cells in the fundus and G cells in the antrum. Studies have shown that acid secretion in response to histamine and pentagastrin is accompanied by a dose-dependent increase in somatostatin secretion, and that somatostatin release is largely determined by intraluminal acidity. It has been proposed that acid-induced release of
somatostatin in proximity to parietal cells serves as a negative feedback mechanism restraining acid secretion.\textsuperscript{33} Somatostatin also has inhibitory effects on the gastrin-releasing G cells.\textsuperscript{23}

Small intestinal distention or acid in the proximal small intestine can initiate reflexes that inhibit gastric secretion.\textsuperscript{16} The presence of acid, fat, or protein breakdown products in the small intestine can stimulate release of peptides such as secretin, and cholecystokinin,\textsuperscript{16} which inhibit acid secretion. Other peptides released from the small intestine that can suppress acid secretion in the stomach include gastric inhibitory peptide, enteroglucagon, and peptide YY.\textsuperscript{18}

**Pepsincogen Secretion**

Chief cells at the base of the gastric gland secrete pepsinogens. Smaller quantities of pepsinogens are also found in mucous neck cells, pyloric glands in the antrum, and cardiac glands.\textsuperscript{36} Pepsinogens, which have no capacity themselves to break down proteins,\textsuperscript{19} are proenzymes of the proteolytic enzyme pepsin, and are composed of proteinases. Following synthesis, pepsinogen is stored in granules within the chief cell. After cell stimulation, the granules fuse with the apical membrane and release their contents into the lumen.
In humans, acetylcholine, histamine, and secretin directly stimulate pepsinogen secretion via increases in intracellular calcium and cyclic AMP. Somatostatin, prostaglandin E₂, and cholecystokinin have been found to decrease pepsinogen secretion.

As intragastric pH decreases due to secretion of hydrochloric acid, pepsinogen is converted to active proteolytic pepsin. The conversion of pepsinogens occurs at a pH below 3.5 and the optimal pH for proteolytic action of pepsin ranges from 2 to 3. Pepsin cleaves proteins at aromatic amino acid links, reducing them to a mixture of peptides.

**Gastric Emptying**

The physical composition of gastric contents is the major determinant of gastric emptying. Solid food remains in the stomach longer than liquid, and the greater the volume of gastric contents, the faster the rate of emptying. In humans, carbohydrates are emptied faster than proteins and fats. Mucosal receptors in the proximal duodenum are sensitive to physical properties and chemical composition of chyme such as pH, osmolarity, and fatty acid content. Gastric emptying is inhibited when fats and acid are present in the duodenum, and conversely, emptying rate is enhanced with increased pyloric diameter and duodenal relaxation. Gastrointestinal
peptides that decrease gastric emptying include secretin, CCK, GIP, and neurotensin.\textsuperscript{22} Vagal innervation is also a necessary component of normal gastric emptying.\textsuperscript{22}

**PATHOPHYSIOLOGY OF GASTRIC ULCERATION**

Acid-peptic disorders account for a major proportion of gastrointestinal disease in humans, and can affect the esophagus, stomach, and duodenum.\textsuperscript{22} Ulcer disease ranges from superficial mucosal lesions (erosions) to deep, even perforating ulcers.\textsuperscript{22,38} The pathogenesis and etiology of ulcer disease are very complex, and not likely to be caused by one abnormality or defect.\textsuperscript{38} Numerous factors have been implicated in human ulcer development, including genetic, neural, hormonal, and iatrogenic components.\textsuperscript{39} Ulcer disease can be related to an imbalance between aggressive factors and the defense and reparative processes of the stomach.\textsuperscript{40} The digestive activity of acid and pepsin are considered aggressive factors, and peptic ulceration does not occur in the absence of gastric acid.\textsuperscript{22} The healing of ulcers in response to acid suppression by histamine type 2 receptor antagonists emphasizes the importance of acid in ulcer pathophysiology.\textsuperscript{40}

The differences in gastric anatomy and physiology between the human and the horse imply that the pathophysiologic processes
of gastric ulceration are not completely shared by both species. There have been many experimental models of gastric ulceration developed in animals, including mucosal surgical resection, thermal, laser, or cryoinjury, and application of acetic acid and ethanol, but these models are not always relevant to naturally occurring peptic disease in humans or horses. A protocol developed recently for inducing gastric ulceration in the horse has provided useful information regarding acid injury to alimentary squamous epithelia in that species.

Mechanisms of Esophageal Mucosal Injury

The equine gastric stratified squamous epithelia has no secretory function, and has less protection against acid injury than the glandular mucosa. The squamous epithelium of the equine stomach shares features with the esophageal epithelium, and it is possible that both structures share common mechanisms for protection against acid. The equine gastric squamous epithelium is probably more susceptible to acid injury than the human esophagus, since it likely has even fewer defense mechanisms. In the horse, gastric ulcers occur most often in the squamous epithelium, therefore it is appropriate to discuss applicable findings regarding mechanisms of injury and esophageal defense in humans and laboratory animals.
Gastroesophageal reflux is a common disorder in humans, and hydrochloric acid is the most important agent initially responsible for injury to the epithelium. Two possible routes of acid entry into the epithelium are transcellular and paracellular pathways, and it is believed that the paracellular path is the major route by which luminal acid damages the esophageal squamous epithelium. Studies have shown that the response of the esophageal epithelium to acid is dependent on acid concentration and time of exposure. At the first stage of acid injury to the esophageal mucosa, there is an increased epithelial passive permeability, where H+ renders cell membranes more permeable to sodium. The result is increased solute and water transport across the epithelium. This is characterized morphologically by dilation of intercellular spaces, which is reversible at this point if acid exposure is discontinued. As damage occurs to cell junctions and apical membranes, hydrogen ion penetrates the epithelium. Hydrogen ion concentrations increase and cause inhibition of the sodium-potassium ATPase on cell membranes. Increased sodium entry into the cell combined with the inability of the ATPase to effectively move sodium out of the cell causes swelling and cell rupture. Necrosis, edema, and vesicle formation are most pronounced in the midzone of the stratum spinosum, where sodium-potassium ATPase sites are most numerous. The morphologic result is midzone cleavage
and detachment of the upper epithelial layer, accompanied by necrosis of the lower layers and ulceration.\textsuperscript{43}

\textbf{Esophageal Mucosal Protection}

The squamous mucosa of the esophagus lacks a well-defined mucus layer like that found in glandular mucosa of the stomach.\textsuperscript{48} Salivary mucin combined with phospholipids form a viscous, hydrogen ion-repelling complex that can be carried along the esophageal mucosa, and with saliva, may help to strengthen the mucosal barrier.\textsuperscript{49} Until recently, it was believed that the esophagus was unable to secrete bicarbonate ions as a defense mechanism. The human esophagus, as well as that of several mammals, has been shown to secrete a bicarbonate-containing fluid with the capacity to clear acid from the lumen, although the likely source of the bicarbonate is submucosal glands, not the squamous epithelium.\textsuperscript{50,51} Submucosal glands are not present in equine gastric squamous epithelia and there is no known bicarbonate secretion.

Without a well-defined mucus layer, the primary esophageal mucosal defense factors are structural and functional elements of the epithelium.\textsuperscript{48} Structural elements include the cell membrane hydrophobic lipid bilayers, and the intercellular barrier to paracellular permeability known as the junctional complex.\textsuperscript{48} The junctional complex consists of the
intercellular tight junction as well as intercellular mucin-like glycoconjugates, and this complex constitutes the major barrier to back-diffusion of luminal hydrogen ions.\(^{48,53}\) Tight junctions have been identified mainly in the stratum corneum of the squamous epithelium, with less being found in the upper layers of the stratum spinosum, and none in the deeper layers. The neutral and acidic glycoprotein material is localized to the stratum corneum and the intercellular spaces for the upper 5-10 layers of the stratum spinosum. This intercellular material, which stains strongly with PAS (Periodic Acid Schiff), appears to be synthesized within cells of the upper epithelium and packaged into membrane-bound vesicles, and is then secreted into the intercellular space.\(^{52}\) Gastric squamous epithelium from foals greater than 2 days old have shown the presence of acidic intercellular mucosubstances with positive PAS staining in the superficial epithelial layers,\(^{53}\) which may serve a similar protective function as the glycoprotein identified in the esophagus.

The functional protective elements of esophageal squamous epithelia include intracellular buffering of hydrogen ions, sodium/hydrogen membrane exchange, and intercellular buffering by bicarbonate.\(^{48}\) Blood flow, which increases during acid exposure, has been shown to be important in preventing acid injury by delivering bicarbonate for intercellular buffering
and maintaining interstitial acid-base balance.\textsuperscript{48}

Another defense mechanism against ulceration is the ability of the epithelium to replicate and repair injury.\textsuperscript{48} Restitution by cellular migration like that seen in the gastric glandular mucosa is not known to occur in the squamous epithelium of the esophagus,\textsuperscript{48} therefore repair is the result of cellular proliferation. Components of gastric juice directly stimulate proliferative activity in the esophageal basal cell layers.\textsuperscript{54} Hydrochloric acid applied to the canine esophagus for 30 minutes caused increased DNA activity and significant mitosis in the basal layer within 20 hours.\textsuperscript{54} Gastroesophageal reflux in human patients causes basal cell hyperplasia of the esophageal epithelium.\textsuperscript{55}

Growth factors are also important in protection of the esophageal squamous epithelium. Salivary epidermal growth factor appears to be important in maintaining the ability of the esophageal mucosa to counteract a high gradient of luminal hydrogen ion.\textsuperscript{49} In one study, removal of salivary glands in rats resulted in a 108\% increase in permeability of the esophageal mucosa to hydrogen ion. Concurrently, there was an 83\% decrease in the mucus content (salivary mucin-lipid complex) on the surface of the mucosa. Supplementation of oral EGF significantly decreased epithelial permeability to
hydrogen ions in sialoadenectomized rats. Recently, one study reported possible esophageal secretion of epidermal growth factor into the lumen, and that the concentration of the growth factor was increased following the introduction of pepsin.56

Gastric Mucosal Protection
There is a million-fold concentration gradient attempting to drive luminal acid into the gastric mucosa, therefore there are multiple structural, functional, and vascular elements of the mucosa that act to protect the stomach from the digestive actions of gastric juice.57 Most of these factors offer protection to the glandular mucosa, but not the squamous epithelium of the equine stomach.

A major deterrent to back-diffusion of luminal acid is the mucus layer, which is adherent to the gastric glandular mucosa. It is composed of glycoprotein from surface mucus cells and mucus neck cells, which forms a gel with water.57,58 The mucus layer entraps bicarbonate produced by gastric surface epithelial cells, and creates a pH gradient to neutralize acid. Surface hydrophobicity is also related to the presence of an intact mucus layer, and is an important barrier property of the stomach.57 The squamous epithelium of the equine stomach has no mucus layer for mucosal protection.
Cellular restitution is an inherent property of the gastrointestinal tract to repair superficial defects caused by topical irritants. Following injury, there is rapid migration of undifferentiated stem cells and mucous neck cells from the gastric pits to cover the basement membrane, and the mucosa can restitute itself in as little as 30 minutes. This process involves migration of cells only and should be differentiated from mucosal regeneration, in which cell division and proliferation occur.

Prostaglandins are potent inhibitors of basal and stimulated gastric acid secretion, and are capable of preventing the formation of gastroduodenal ulcers. The ability of prostaglandins to protect the gastric mucosa is independent of their ability to inhibit acid secretion, and virtually all prostaglandin types have demonstrated the ability to protect against ulceration. Prostaglandins, particularly PGE₂, also prevent disruption of the mucosal barrier, maintain gastric mucosal blood flow, and stimulate mucus and bicarbonate secretion.

Growth factors, particularly epidermal growth factor and transforming growth factor alpha, are believed to play important roles in maintenance of the gastric mucosa. Both peptides are potent inhibitors of gastric acid secretion when
administered parenterally.\textsuperscript{3} EGF has been shown to cause increased DNA synthesis and cellular proliferation,\textsuperscript{64} as well as acceleration of gastric ulcer healing.\textsuperscript{5}

Other gastric mucosal defense mechanisms include apical membrane integrity, tight junctions between epithelial cells, sulfhydryl groups, muscle tone of the stomach, gastric emptying, intracellular bicarbonates, and transmembrane ion exchanges.\textsuperscript{29,61,65}

\textbf{Etiologic Factors in Ulcer Disease}

Gastric acid is still considered to be a major prerequisite in peptic ulcer disease in humans and horses.\textsuperscript{11,66,67} In general, gastric ulceration in humans is more associated with decreased tissue resistance to peptic secretions than with increased acid and pepsin secretion.\textsuperscript{66} Human gastric ulcer patients actually have decreased mean basal and stimulated acid outputs when compared to normal, suggesting that factors other than acid/pepsin injury are important.\textsuperscript{67}

\textit{Helicobacter pylori} is currently considered to be a primary etiologic agent of most peptic ulcer disease in humans.\textsuperscript{40} \textit{H. pylori} is found in 60-80\% of human gastric ulcer patients and 95-100\% of those with duodenal ulcers.\textsuperscript{40,57} The bacteria are contained within the mucus coat of the gastric antrum, and
appear to have mucolytic and proteolytic properties which degrade the mucus barrier.\textsuperscript{57} It has been postulated that \textit{H. pylori} also elaborates phospholipase enzymes that hydrolyze phospholipids responsible for surface hydrophobicity.\textsuperscript{57} Gastric ulcers in humans are commonly associated with gastritis, and \textit{H. pylori} is the major determinant of chronic active gastritis.\textsuperscript{40} \textit{H. pylori} only lives in gastric epithelium, and is thought to colonize gastric metaplasia in the duodenum.\textsuperscript{40} \textit{H. pylori} has not been found in normal or ulcerated gastric/duodenal mucosa from foals,\textsuperscript{69} and has not been identified in any equine species.\textsuperscript{11} In humans, the gastritis and associated lesions caused by \textit{H. pylori} infection result in an inflammatory response,\textsuperscript{40} whereas glandular lesions in horses tend to lack pronounced inflammation.\textsuperscript{70} Based upon current information, it would appear that \textit{H. pylori} is an unlikely etiologic factor of peptic ulcer disease in the horse.

In adult horses, gastric ulcers occur most frequently in the stratified squamous epithelial mucosa, and ulceration is considered to be the result of excessive exposure to hydrochloric acid.\textsuperscript{8,11} Typically, the squamous epithelium of the equine stomach is not exposed to a highly acidic pH.\textsuperscript{28} The median 24 hour gastric pH was significantly more acidic in horses withheld from feed (3.1 in fed horses vs 1.5 in unfed)
and prolonged increased gastric acidity that occurs when horses are deprived of feed damages the gastric epithelial mucosa.\textsuperscript{28} This effect is elegantly demonstrated in a report in which gastric squamous epithelial ulceration was induced in horses by exposing the gastric mucosa to repeated periods of high acidity.\textsuperscript{41} Horses with endoscopically normal stomachs were fed free choice hay for 24 hour periods alternating with 24 hour periods during which hay was withheld. With this protocol, progressive erosion and ulceration of the gastric squamous mucosa occurred in 9/10 horses after a total of 7 days (84 hours off-feed). With feed deprivation, mucosal damage can occur within a matter of hours,\textsuperscript{70} and as demonstrated by the horses in this study,\textsuperscript{41} 7/10 horses had erosions after only a total of 36 hours off feed. The pH gradient of the equine stomach from dorsal to ventral appears to be relevant to lesion location, as the pattern of squamous ulceration reflects the degree of exposure of the epithelium to acid.\textsuperscript{30,41} Ulcers induced in this study were most severe adjacent to the margo plicatus, particularly along the right side and lesser curvature of the stomach,\textsuperscript{41} which anatomically should have the greatest contact with gastric acid. Lesions decreased dorsally, where mucosal surface pH is typically higher,\textsuperscript{30} and there were no lesions in the most dorsal portion of the stomach. No ulceration occurred in the gastric glandular mucosa at any time during the feed deprivation
It is evident that the time a horse spends eating is an important factor in the development of squamous ulceration. Consumption of feed has a neutralizing effect on gastric acidity by salivary bicarbonate as well as bulk absorption of gastric fluid. Horses that are maintained on pasture and allowed to graze continuously usually have no gastric ulceration, illustrating that feeding management and eating behavior are likely to be paramount in determining whether squamous ulceration occurs in the horse. Little is known regarding the effect of various feedstuffs on acid secretion in the horse, although it has been shown that consumption of grain causes higher serum gastrin levels than feeding grass hay.

Gastric Ulcer Disorders in Horses and Foals

Gastric ulceration is frequently seen in foals and horses. The presence of gastric lesions does not always correlate with clinical signs, therefore definitive diagnosis is made only by direct endoscopic examination or post-mortem evaluation. Three recent endoscopic surveys of asymptomatic foals determined that 50% of foals (mean ages 23, 28, and 40 days) had gastric ulcers. Lesions in the asymptomatic foal are found mainly in the squamous epithelial mucosa, and the
prevalence is greatest in foals less than 50 days of age. Lesions occur in the squamous mucosa adjacent to the margo plicatus along the greater curvature. In older foals, lesions occur more commonly along the lesser curvature or cardia and are more diffusely distributed.  

Gastric ulceration is common in adult horses and may result in poor performance, chronic colic, poor body condition and decreased appetite. Ulcers are seen frequently in the squamous epithelium, particularly adjacent to the margo plicatus and along the lesser curvature. A greater prevalence and severity of gastric lesions have been found in horses in race training than those not in training, with 76% of asymptomatic racehorses having ulceration compared to 37% of asymptomatic pleasure or show horses. A post-mortem study of Thoroughbreds in Hong Kong similarly showed that 80% of racehorses in training had ulceration compared to 52% of racehorses that had been out of training for a month or more. Gastric lesions in racehorses appeared to be progressive and became more severe during continued training.  

There is an association between clinical signs and gastric ulceration, based upon a greater prevalence and severity of ulcers in symptomatic versus asymptomatic horses. In 87 horses with clinical signs, 92% had gastric ulceration, with
lesion severity being significantly greatest at the margo plicatus. In a study of 111 horses with colic, 82% had gastric ulceration, and 63% of those horses had no other detectable abnormalities involving the abdominal viscera. It is therefore reasonable to conclude that gastric ulceration is of clinical significance in the adult horse as well as the foal.

CHARACTERISTICS OF GASTROINTESTINAL MUCOSAL HEALING

Gastrointestinal tract mucosa has one of the most rapid turnover rates of any tissue in the body, and like bone marrow and skin, is designated as a constantly renewing cell population. The cellular mechanics of routine gastrointestinal renewal are relevant in the healing of mucosal defects, as both processes involve proliferation, migration, differentiation and maturation of cells to maintain an intact mucosa. Typically, the populations of dividing and maturing cells remain in a steady state and are balanced with cell loss. Logically, increased loss of cells will result in cell population changes.

In general, gastrointestinal cell renewal is initiated in proliferative cell zones, where undifferentiated cells divide, and in most instances, migrate towards the gut lumen where they are eventually lost. As the cells migrate toward the
lumen, they become differentiated to perform particular functions and the ability to divide is lost. The cell renewal cycle is composed of the mitosis (M) phase, the postmitotic, presynthetic $G_1$ phase, the DNA synthesis (S) phase, and the postsynthetic, premitotic $G_2$ phase. In the $G_1$ phase, some cells may enter the "resting" $G_0$ phase, where cell proliferation is possible but is temporarily suspended. After the mitosis phase, cells that don’t continue in the cell cycle differentiate then move luminally in the epithelium. The time intervals of the cell cycle phases may vary in different sections of the gastrointestinal tract, but in general, the M phase is one hour, followed by the $G_1$ phase at 10-15 hours. The S phase of DNA synthesis lasts about 10 hours and the $G_2$ phase 1-6 hours, for a total cycle time of one to two days.

In the stratified squamous epithelium of the esophagus and the equine stomach, the proliferative zone is the basal layer of progenitor cells which are applied to the basement membrane. The progenitor cells divide and can leave the basal layer to differentiate, or they can remain as basal cells and divide again. Above the basal layer are layers of nonproliferative cells that progress towards the lumen and become more flattened in appearance. In rodents, renewal of the basal layer of the esophageal squamous epithelium takes about 4-5 days, with replacement of the epithelium taking 7 days.
humans, esophageal mucosal replacement takes longer, approximately 2 weeks.\textsuperscript{82}

Gastric erosion and ulceration of the glandular or squamous portions of the equine stomach involve damage to the mucosa which is repaired by cellular proliferation. Once a gastric erosion or ulcer develops, similar common stages of repair take place.\textsuperscript{39} These stages are like those described for wound healing of the skin, and comprise a complex interaction of many cell types that are influenced by inflammatory mediators, chemotactic factors, angiogenic factors, and growth factors. The mechanics of healing is dependent upon whether the lesion is an erosion or an ulcer, and if the lesion is located in the squamous versus the glandular epithelium of the stomach. An ulcer occurs when all epithelial layers are lost and the lamina propria is exposed to the lumen, whereas an erosion has intact epithelium still present over the lamina propria. General overlapping stages of wound repair include an inflammatory and debridement stage, a repair stage, and a maturation/remodeling stage.\textsuperscript{83}

The inflammatory stage is characterized by loss of epithelium and subsequent vasodilation, increased vascular permeability, submucosal edema, and concomitant platelet aggregation.\textsuperscript{65,84} Platelet granules deliver factors crucial to the healing
process that include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor alpha and beta (TGF), and fibroblast growth factor (FGF). Neutrophils adhere to the endothelium, pass through junctions between endothelial cells, then infiltrate the ulcer to begin phagocytizing debris. Leukocyte chemoattractants such as leukotriene B₄ and complement C₅a may act to draw more neutrophils during the inflammatory response. Monocytes invade the wounded area shortly thereafter, and this accumulation of mononuclear cells that become macrophages is considered critical to initiation of tissue repair. Macrophages remove tissue debris and subsequently release cytokines that are necessary for stimulating the formation of granulation tissue, including PDGF, TGF-alpha and beta and FGF. PDGF is chemotactic for macrophages and is a powerful mitogen for fibroblasts. EGF and TGF-alpha are important in stimulating mitosis, cellular proliferation, and epithelial migration. TGF-beta stimulates fibroblast proliferation and collagen synthesis, and is chemotactic for inflammatory cells and fibroblasts. FGF is a potent angiogenic factor, and is essential in neovascularization and formation of granulation tissue.

The repair stage involves angiogenesis, granulation tissue formation, mucosal proliferation, and wound contraction.
Angiogenesis is crucial for the healing process, and occurs simultaneously with fibroplasia.\textsuperscript{85} It is an extremely complex process that depends upon mitogenic factors as well as endothelial cell chemotactic factors. Vascular ingrowth occurs following the infiltration of leukocytes during the inflammatory stage, although neovascularization has been shown to occur even with leukocyte depletion.\textsuperscript{85} This implies that there are many factors that influence the initiation of angiogenesis. Microvascular endothelial cells are stimulated to migrate into the wounded area and to proliferate, forming new capillaries. In ulcerated gastric epithelium, this angiogenesis occurs in the lamina propria.

The mucosa at the ulcer margin is highly proliferative, with increased thickness of all epithelial layers. This proliferation provides cells for re-epithelialization.\textsuperscript{88,89} The mucosa at the ulcer margin is considered to play a major role in the healing process, and acts as a transitional zone between ulcerated and normal tissue.\textsuperscript{39}

Granulation tissue composed primarily of fibroblasts, endothelial cells, and macrophages in a bed of collagen and fibronectin is formed over the ulcer bed.\textsuperscript{90} This tissue is important in providing a framework and supplying cells for restoration of the lamina propria, as well as for initiating
neovascularization that will deliver oxygen and other nutrients to the ulcer base.\textsuperscript{39} In the granulation tissue, there is proliferation of lamina propria myofibroblasts, which are modified fibroblasts with characteristics of smooth muscle cells.\textsuperscript{85} Wound contraction occurs as the myofibroblasts develop attachments to the wound edges and bed, and by traction forces, draw the ulcer edges closer together.\textsuperscript{83,85}

In the maturation or late stages of healing, there is continued mucosal proliferation and contraction of the lesion. Further fibroplasia and collagen realignment take place, and inflammation resolves. As healing occurs and the need for increased blood flow diminishes, there is capillary regression.\textsuperscript{85}

Healing of gastric squamous ulcers occurs primarily by epithelial proliferation and wound contraction. For erosions, where the basal layer of epithelium is still intact, there is basal cell and erosion margin proliferation, which allows healing to occur primarily by re-epithelialization.\textsuperscript{76} Histologic studies of the normal stratified squamous epithelium of the esophagus have shown that the basal zone thickness is approximately 10\% of the total epithelial thickness and papillae extend one-half the distance to the epithelial surface. In human patients with gastroesophageal
reflux, basal cell hyperplasia occurred, with mean basal zone thickness increasing to 30% and papillae extending more than two-thirds of the distance to the epithelial surface. These changes were thought to occur due to increased rate of epithelial cell replacement.55

Topical growth factors applied to epithelial surfaces have been shown to improve healing.4,7,86 PDGF, FGF, and EGF applied topically to skin have been reported to enhance the rate of granulation tissue formation,7 and PDGF and TGF-beta have been shown to increase fibroplasia and neovascularization of full-thickness wounds in mice.66 Topically applied EGF accelerates epidermal regeneration in split-thickness wounds and partial-thickness burns,6 was well as enhances healing of chronic corneal ulcers in dogs.7 An FGF analog has also been shown to accelerate the healing of experimentally-induced duodenal ulcers in rats.7 The beneficial effects of exogenously administered growth factors demonstrates the enormous complexity of the wound healing process, and that there may be potential for clinical application of these agents.

GROWTH FACTORS

Introduction

Growth factors are important regulatory substances in the body, and our understanding of their role in stimulating cell
proliferation and differentiation is still limited. There are many different growth factors, including epidermal growth factor (EGF), transforming growth factor (TGF) alpha and beta, fibroblast growth factor (FGF), platelet derived growth factor (PDGF), and insulin-like growth factor (IGF) that are polypeptide hormones involved in physiologic and pathologic processes, including embryogenesis, growth, tissue repair, regeneration, and neoplasia. Growth factors are produced and secreted by cells from a wide variety of tissues, and exert their actions by binding to specific cell surface receptors and initiating postreceptor signal transduction mechanisms. Most growth factors stimulate cellular proliferation, although there are some polypeptides that inhibit proliferation. Growth factor effects are seen at ng/ml concentrations.

Growth factor effects occur most frequently through paracrine and autocrine mechanisms, although some growth factors may act as hormones and be carried to distant target cells in plasma. The autocrine pathway allows the growth factor to regulate cellular function by binding to receptors on the membrane of the same cell that synthesized and secreted the growth factor. Paracrine regulation occurs when the growth factor binds to receptors located on adjacent cell membranes.
EPIDERMAL GROWTH FACTOR

Sources and Body Distribution

Epidermal growth factor was isolated in 1962 by Stanley Cohen, and has been the subject of extensive research efforts for the last 30 years. Many of its mechanisms of action still remain a mystery. Epidermal growth factor is found in human and animal tissues as well as body fluids, although the sites of synthesis remain uncertain in most species. Radioimmunoassay in humans has detected and quantitated immunoreactive EGF distributed in tissues from virtually all organ systems throughout the body. Large quantities of EGF have been found in the human salivary gland, kidney and thyroid gland, and immunohistochemical staining for EGF in human tissues has shown reactivity mainly in epithelial cells of the lung, stomach, duodenum, pancreas, kidney, pituitary gland, thyroid gland, mammary gland, ovary, uterus, and placenta.

The major sources of EGF in the human body are the salivary glands, Brunner’s glands in the duodenum, kidneys, and the pancreas. In salivary and pancreatic tissues, EGF is stored in granules in the tubular or ductal cells and secreted into saliva and pancreatic juice. Other body secretions that contain EGF include urine, gastric juice, bile, sweat, prostatic fluid and milk. This would suggest possible production of EGF by fluid-secreting cells or transport of EGF
from plasma into secretions.91

Epidermal growth factor is present in many tissues, but is not manufactured by all of the tissues where it is found. Expression of EGF messenger RNA in humans has been detected in the salivary gland, kidney, mammary gland, and thyroid gland.94 Very low levels of EGF mRNA have also been found in the pancreas and small intestine.92 Extensive immunoreactivity of EGF in other body tissues is possibly the result of temporary storage in those cells, rather than actual EGF production.94 It is also possible that EGF is produced in other tissues but the mRNA may be present in low levels that have not been detectable.

In comparison to other tissues, the level of EGF in the plasma is very low, possibly due to its short half-life and rapid removal by various tissues, particularly the liver.96 It can exist as a free plasma protein in the bloodstream, or bound to platelets.2 The source of circulating plasma EGF in the human remains unknown, with possible origins being the salivary gland and kidney.96 In one report, human plasma EGF level and salivary output of EGF were significantly increased for 30 to 60 minutes over basal EGF concentrations following modified sham feeding and regular feeding,96 suggesting that the salivary gland may be a primary source of plasma EGF. The
kidney could also be the main source of EGF in human plasma, due to the high level of EGF mRNA expressed in that organ. The liver regulates the circulating level of EGF, where it can be degraded in hepatocytes and secreted into bile. Up to 20% of EGF secreted in bile has been found to be intact EGF that can be taken up again by hepatocytes.

Factors Affecting Release of Epidermal Growth Factor

The release of EGF is thought to be controlled by complex neurohormonal mechanisms, many of which remain to be elucidated. Alpha adrenergic stimulation and vasoactive intestinal peptide (VIP) have been shown to affect secretion of EGF from Brunner's glands in the rat, and alpha adrenergic agents regulate release of EGF from the submaxillary salivary gland in mice. Androgenic stimulation will cause an increase in plasma EGF, and this has recently been reported in humans following intensive and prolonged exercise, and in mice after aggressive behavior. Infusion of pentagastrin has been shown to significantly increase EGF release in saliva and duodenal secretions, whereas somatostatin will inhibit EGF release.

Epidermal Growth Factor Structure

In the mouse and human, EGF is a 53 amino acid, low molecular weight polypeptide of approximately 6,000 Da. There are
variations in the number of amino acids and their sequence from one species to another. Transforming growth factor-alpha (TGF-alpha) is capable of binding to the EGF receptor and shares similar structure.\textsuperscript{101} Other peptides considered members of the EGF-related family are amphiregulin (AR), heparin-binding epidermal growth factor (HB-EGF), and betacellulin.\textsuperscript{92} There are consistently six cysteine residues in all of these molecules which are arranged as identical disulfide bonds, and the preservation of these cysteine residues suggests that they are probably important in receptor binding.\textsuperscript{93,102} Because of the conservation of these cysteine(C) residues and invariant glycine(G) residues, similarities have been noted between the EGF family and complement proteins Factor IX, Factor X, Protein C, plasminogen activator, and fibronectin. All have an amino acid sequence of CxCxxGxxGxxC.\textsuperscript{93}

In mice, EGF mRNA consists of 4800 nucleotides and codes for a 1,217 amino acid EGF precursor molecule called prepro EGF.\textsuperscript{91,102} The precursor is 24 times larger than mature EGF, and consists of a 25 amino acid signal peptide, seven EGF-like structures, EGF itself, and a 21 amino acid hydrophobic carboxyterminus.\textsuperscript{91} The precursor is thought to be produced extracellularly on the cell membrane, and the carboxyterminus of the molecule serves as a membrane anchor, directing the insertion of prepro EGF into the cell membrane.\textsuperscript{93,101,103} The
precursor is then cleaved by proteases to form the mature peptide.\textsuperscript{101,103} It is not known why the precursor molecule is so large, or what happens to the seven EGF-like proteins encoded by the homologous repeats.\textsuperscript{102} The repeat EGF-like sequences contained in prepro EGF have alterations in the highly conserved cysteine residues as well as the other invariant amino acid positions, therefore these peptides would unlikely be capable of binding to the EGF receptor.\textsuperscript{102}

Similarities have been noted between portions of prepro EGF and the light density lipoprotein receptor molecule (LDL), and it has been suggested that the EGF precursor could also function as a receptor by binding an unidentified ligand at its N-terminal region.\textsuperscript{91,102} It is also thought that, as a transmembrane protein, prepro EGF could provide an immobilized growth factor that is capable of stimulating adjacent cells.\textsuperscript{101} The biologic function of the EGF precursor requires further investigation.
FIGURE B: Structure of epidermal growth factor

Amino acid sequence with placement of disulfide bonds between cysteine residues. (Reprinted from Journal of Biological Chemistry 1973; 248: 7669-7672)
FIGURE C: Structure of the epidermal growth factor precursor

Mouse prepro-EGF attached to the cell membrane. The seven EGF-like peptides are cleaved off by proteases to form the mature peptide. (From Marti, Burwen, and Jones. Biologic Effects of Epidermal Growth Factor. Hepatology 1989; 9(1): p 126-138)
Epidermal Growth Factor Receptor

The EGF amino acid sequence may vary from one species to another, but all EGF molecules are capable of binding to the same EGF receptor. EGF receptors are found in most all mammalian species\textsuperscript{93} and have a wide tissue distribution, especially epithelia.\textsuperscript{104} It has been estimated that there may be up to 100,000 EGF binding sites on a single cell,\textsuperscript{91} and the only cells that have not been found to possess EGF receptors are those of the hematopoietic system.\textsuperscript{93} EGF receptors have been documented in the gastrointestinal tract of humans,\textsuperscript{103} laboratory animals,\textsuperscript{105} dogs,\textsuperscript{106} pigs,\textsuperscript{107} and horses.\textsuperscript{108}

In the stratified squamous epithelium of the oral cavity, esophagus, and equine and porcine squamous epithelial gastric mucosa, receptors are localized to the basal layers and diminish toward the mucosal surface.\textsuperscript{103,107,108,109} EGF receptor messenger RNA is expressed in the basal layer of squamous epithelium.\textsuperscript{110} In the glandular mucosa of the stomach, receptors have been localized to the basolateral and apical membranes of mucous neck cells in the gastric pit proliferative zone,\textsuperscript{103} and the basolateral membranes and cytoplasm of parietal cells.\textsuperscript{111}

The mature EGF receptor is a transmembrane glycoprotein that consists of 1,186 amino acids.\textsuperscript{91} There is an N-terminal,
extracellular EGF-binding domain of 621 amino acids, a transmembrane sequence of 23 amino acids, and a carboxyterminal, intracellular (cytoplasmic) domain consisting of 542 amino acids, all having a mature molecular weight of 170 kD.\textsuperscript{112}

The binding of EGF to its receptor initiates a cascade of poorly-understood events that result in the transmission of a mitogenic signal. After EGF binds to the external receptor domain, a tyrosine protein kinase is activated, which phosphorylates the EGF receptor itself as well as other substrates.\textsuperscript{91} The kinase and the EGF-binding activity have subsequently been determined to exist in different domains within the same EGF receptor molecule.\textsuperscript{104,113} The structure of the EGF receptor kinase resembles the sequences described for other tyrosine kinases,\textsuperscript{93} and is a 170 kD protein located in the cytoplasmic domain of the receptor.\textsuperscript{113} The three tyrosine residues that are autophosphorylated by the kinase are located at amino acid positions 1058, 1148, and 1173 of the EGF receptor,\textsuperscript{93} and are contained in a 20 kD segment near the cytoplasmic carboxyterminus.\textsuperscript{112,113} The mechanism by which EGF-binding activates the protein kinase activity is unknown.\textsuperscript{93,113}

The EGF/receptor complex is ultimately internalized and degraded in lysosomes.\textsuperscript{113} Following binding of EGF at the cell
surface, the receptors cluster into aggregates in depressions of the plasma membrane.\textsuperscript{114} The clusters are then rapidly internalized into endocytic vesicles,\textsuperscript{113} and are passed through an intracellular endosome system. Partial proteolysis of EGF may occur in the vesicles, with final proteolytic degradation taking place at the mature lysosome.\textsuperscript{1} In cell fractionation studies, labelled EGF was found at the same gradient position as lysosomes within only 30 minutes of internalization into the cell,\textsuperscript{114} emphasizing how rapidly EGF reaches the lysosome for degradation. There is evidence that most receptor complexes are transferred through the endosome system and are not recycled back to the cell membrane.\textsuperscript{114}

Internalization of EGF can cause a decrease in the number of cell surface EGF receptors, a process known as down regulation.\textsuperscript{114} Low concentrations of EGF causing steady-state receptor occupancy can result in a 30-40\% reduction in binding activity,\textsuperscript{104} and cells are thought to be capable of down-regulating the surface receptor population by 50\% within 1-2 minutes of EGF binding.\textsuperscript{114} Internalization and subsequent downregulation of the human EGF receptor is thought to be controlled by the carboxyterminal tyrosine residues of the EGF receptor molecule.\textsuperscript{115}
FIGURE D: Structure of the epidermal growth factor receptor

EGF receptor attached to the cell membrane, illustrating the extracellular, transmembrane, and intracellular components. The 24-amino acid signal sequence is cleaved off, leaving the 1,186 amino acid mature receptor. (From Marti, Burwen, and Jones. Biologic Effects of Epidermal Growth Factor, with Emphasis on the GI Tract and Liver. Hepatology 1989; 9(1): p 126-138)
As EGF and its receptor are processed within the cell, generation of a mitogenic signal occurs that ultimately results in DNA synthesis and cell division. The significance of the events following the internalization of EGF are unknown, and it is also not known which events are essential for initiation of the mitogenic signal. Brief exposure of cells to EGF does not result in mitogenesis, and EGF must be present in cell medium for a minimum of 5 hours for any DNA synthesis to occur. A 12-15 hour exposure to EGF has been found to elicit maximal DNA synthesis, which occurs at 24 hours following exposure. It is likely that multiple signals are necessary in the stimulation of DNA synthesis, although the intracellular mediators and second messengers remain largely unknown. Studies have indicated that aggregation and clustering of the receptor, as well as phosphorylation of the EGF receptor generate at least two of the required signals for cell replication, but neither is sufficient by itself for induction of DNA synthesis.

Other post-receptor mechanisms that may be involved in signal transduction include modulation of cyclic AMP metabolism, increases in cellular pH and cytoplasmic free Ca\(^{2+}\) levels, metabolism of arachidonic acid to oxygenated metabolites, phosphorylation of intracellular proteins, and enhancement of the membrane-associated Na+-K+ pump. EGF has also been
shown to stimulate the enzyme ornithine decarboxylase (ODC), which plays a key role in the synthesis of polyamines, the regulation of RNA polymerase, and RNA and DNA synthesis.

For DNA synthesis and cell proliferation to occur, cells must be exposed to growth factors at the transition from the M phase (mitosis) into the G1 phase (gap between mitosis and DNA synthesis) of the cell cycle, and through at least the first half of G1. During the postmitotic stage, the cell can begin another replicative cycle or enter the quiescent G0 phase, and once the cells have entered G1, EGF is required for a 10 hour period for cells to re-enter the cell cycle from G1. In a human colonic cell line, exposure to EGF in the S phase (DNA synthesis) of the cell cycle was insufficient for the cells to continue cycling once they reached G1, and if cells were exposed to EGF for only 2 hours in G1, no cells continued to cycle.

Physiologic Effects of Epidermal Growth Factor

General Effects

The biologic actions of EGF are widely diverse throughout the body, and include effects that are not related to mitogenesis. General biologic activity of EGF includes short-term and delayed effects. Short-term effects can occur within seconds or minutes, and include stimulatory and inhibitory effects on
ion and molecular transport. These increases in transport mechanisms can occur before internalization of the receptor complex, and are based on a mechanism at the cell surface. The major delayed effect of EGF is stimulation of DNA synthesis and cell proliferation. Other general effects are enhancement of keratin, fibronectin, and collagen synthesis, as well as prostaglandin synthesis. EGF can also affect the synthesis and release of a variety of hormones, including hCG, ACTH, thyroid hormone, luteinizing hormone, growth hormone, and prolactin. EGF modulates the contractility of isolated arterial strips, and it has been shown to have a potent vasodilatory effect in anesthetized dogs. EGF also may play a role in glucose metabolism, gluconeogenesis and hepatocyte homeostasis.

The salivary glands are presumed to be the major source of EGF for the proximal digestive tract. Proposed physiologic actions important to the gastrointestinal tract include development of the neonatal intestinal tract, stimulation of cell proliferation and differentiation, inhibition of gastric acid secretion, protection of the gastric mucosa against topical irritants, and acceleration of ulcer healing. The actions of EGF are potentially exerted by direct exposure of the mucosa to luminal EGF, plasma EGF carried to receptors via the bloodstream, and local release of EGF that reaches receptors

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via autocrine and paracrine mechanisms.

**Gastrointestinal Development**

EGF has a wide spectrum of biologic activity in the gastrointestinal tract, particularly regarding the neonate. EGF was initially thought to have a primary influence on prenatal development, since EGF receptors have been found in fetal tissue, even as early as a 3-5 day blastocyst.\(^{128}\) Neither EGF or EGF mRNA have been found in the developing fetal mouse, and the EGF-like activity that has been detected is most likely TGF-alpha.\(^{102}\) Immunoreactive TGF-alpha has been detected in the rat distal colon at 18 days of gestation.\(^{129}\) TGF-alpha mRNA is expressed in the maternal decidual cells, and levels are highest in the region adjacent to the embryo.\(^{130}\) It is therefore thought that TGF-alpha has an autocrine influence on decidual growth, and paracrine stimulation on embryonic development by way of the EGF receptor.\(^{102,128}\)

EGF plays an important role in the development of the gut postnatally, and is capable of accelerating maturation and stimulating cellular proliferation.\(^{125}\) It is present in the milk of mammalian species,\(^{131}\) and has been shown to enhance neonatal intestinal growth.\(^{132}\) There is no EGF mRNA in the salivary gland of mice until after weaning,\(^{102}\) and milk is the principal source of EGF in the suckling mouse.\(^{133}\) There are
species variations in the concentration of milk-derived EGF at different stages of lactation.\textsuperscript{131} Species differences in the milk EGF levels may be related to the concurrent degree of gastric acid secretion present in those species, since EGF is important in gastric mucosal protection.\textsuperscript{2}

Immunoreactive EGF has been found in the lumen of the entire gastrointestinal tract of the neonate and the adult. Ingested EGF is absorbed by the intestinal tract of neonates and distributed to the liver, lung, kidney and brain,\textsuperscript{134,135} whereas, EGF is not absorbed from the lumen in adults.\textsuperscript{136} In a study using neonatal rat pups, EGF was provided orally in formula for 5 days at a dose that exceeded the reported concentrations in rodent milk, and those pups had significantly greater body weight, intestinal weight, length, and DNA content than those did not receive exogenous EGF.\textsuperscript{132} Another study showed that rat newborns fed EGF for 39 hours after birth had heavier livers, hearts, and kidneys after 5 days than control rats.\textsuperscript{134}

\textbf{Inhibition of Gastric Acid Secretion}

Intravenous or subcutaneous parenterally-administered EGF and TGF-alpha have been shown to potently inhibit gastric acid and pepsin secretion.\textsuperscript{3,96,126,137} Intragastric administration of EGF or TGF-alpha does not inhibit acid secretion.\textsuperscript{3} This suggests that luminal EGF in the stomach does little to regulate acid
secretion. EGF receptors have been demonstrated on the basolateral membrane of parietal cells, therefore it is possible that the receptors respond to EGF from the systemic circulation. The inhibition of acid secretion after parenteral administration occurs very rapidly, suggesting that the signal from the bound EGF receptors to the parietal cell acid production system does not require continuous exposure to EGF, as does EGF-stimulated DNA synthesis.

The mechanism of EGF-mediated inhibition of acid secretion is not fully understood, but EGF appears to decrease the secretory activity of the parietal cell directly. EGF given intravenously to dogs inhibited gastric acid secretion stimulated by food as well as exogenous stimulants pentagastrin and histamine. Inhibition of acid secretion does not appear to require vagus innervation, and EGF acid inhibition caused no changes in plasma gastrin levels. Studies utilizing isolated rat and rabbit parietal cells have concluded that EGF inhibits histamine-stimulated gastric acid secretion by decreasing the cyclic AMP content of parietal cells.

There is apparently little evidence that normal physiologic levels of EGF in the circulation affect gastric acid secretion. A study using healthy human subjects found that
exogenous, intravenous EGF caused a dose-related inhibition of gastric acid and pepsin secretion related to the level of plasma EGF. A dose of EGF similar to that seen in plasma after feeding did not significantly affect acid secretion, implying that endogenous EGF released into the circulation under these conditions would not noticeably affect gastric secretion. In that study, plasma EGF promptly returned to basal levels after EGF infusion was stopped, accompanied by a decrease in gastric acid output within 45 minutes.

Stimulation of Cell Proliferation

EGF is a potent mitogen and stimulates proliferation of many cell types, especially epithelial cells. Its actions can be modulated by other growth factors such as PDGF, TGF-alpha, and TGF-beta. In tissue cultures, EGF has been shown to stimulate proliferation of fibroblasts, keratinocytes, chondrocytes, glia, and vascular endothelial cells. EGF appears to play an important role as a trophic factor in the maintenance of epithelial proliferation of the gastrointestinal tract, although the route and mechanisms by which it exerts these actions remain speculative. It has been established that EGF’s mitogenic capabilities are independent of its ability to inhibit gastric acid secretion. EGF administered intravenously to rats stimulates epithelial growth and increases intestinal weight in all sites of the
gastrointestinal tract in a dose-dependent manner, especially in the stomach and colon. EGF injected intraperitoneally has also been shown to increase DNA synthesis in the mucosa of the stomach. Intragastric administration of EGF had no effect on intestinal weight or crypt cell production rate, and it has been proposed, based upon these findings, that the maintenance of gastrointestinal growth occurs through a systemic mechanism or local release of EGF rather than from luminal exposure. The actions of EGF may be interrelated with other hormones that are important in trophism of the GI tract, including growth hormone, growth hormone releasing factor, and gastrin.

The role of luminal EGF remains unclear. It has been reported that EGF is not absorbed from the lumen in the adult animal, and there continue to be conflicting reports regarding the location of the receptor in the gastrointestinal epithelium. In the glandular rat stomach, studies have reported EGF receptors predominantly in the mucous neck cells of the proliferative zone along the apical and basolateral cell surfaces, and on the cell membrane and within the cytoplasm of parietal cells. Because the proliferative zone contains progenitor cells that can divide and migrate, it has been assumed that they are primary targets for EGF's mitogenic action. The apical receptor location on the mucous neck
cells leads to the theory that luminal EGF or EGF-like substances would have access to bind these receptors and stimulate mitosis.\textsuperscript{143} The EGF receptors tend to be expressed most prevalently on actively proliferating cells, and as further differentiation of cells occurs, receptor expression becomes decreased.\textsuperscript{144,145,146} In rat epithelia (skin), there is a decrease in basal cell EGF receptor number as growth rate diminishes with age.\textsuperscript{145}

In contrast to the stomach, other studies have reported that EGF receptors in the small intestine are localized only to the basolateral membrane in both the crypt and villus cells of fetal, suckling, and adult rat\textsuperscript{136} as well as human fetal tissue.\textsuperscript{146} It was found that luminal EGF was unable to pass the intact epithelial barrier of the adult intestine to reach the basolaterally located receptor.\textsuperscript{136}

Based upon these findings, one may assume that luminal EGF does not pass through the stratified squamous epithelia of the equine stomach and the human esophagus to reach EGF receptors located on the basal epithelial layers, because these receptors are not in contact with the lumen.\textsuperscript{147} In the human esophagus, EGF has been found in the capillary endothelium adjacent to papillae basal cells, which are in close proximity to the basally-located receptor and the proliferative zone.
This suggests that EGF may reach these receptors via the circulation.148

Protection of the Gastric Mucosa
EGF has been reported to have an important role in protecting the gastric mucosa against various ulcerogens.3,149,150,151 Parenteral doses of EGF that are too low to inhibit acid secretion have prevented the formation of aspirin-induced gastric ulcers.2 The rapidity with which EGF can offer protection to the gastric mucosa would preclude DNA synthesis and increased mitotic activity as a relevant mechanism, since a long latent period is required for those actions to occur.141

Parenteral (intraperitoneal or subcutaneous) EGF in rats provided gastric mucosal protection against water immersion and restraint stress,152 ethanol,3,150 and acid,153, and protective effects were seen in as little as 10–30 minutes following parenteral injection of EGF.3,150,153 Intraperitoneal EGF administered simultaneously with intragastric hydrochloric acid offered no protective effects against mucosal injury.153 Several studies have reported that topical EGF protected the gastric mucosa against ethanol151 and acid,149 whereas a more recent report concluded that intragastric EGF or TGF-alpha applied 30 minutes prior to ethanol application failed to provide any protection against injury.3
Recent studies have concluded that the ability of EGF to protect the gastric mucosa may be mediated by mucosal prostaglandins,\(^3,151,152\) and that this interrelation between EGF and prostaglandins may involve the preservation of mucosal blood flow.\(^3\) EGF has also been reported to protect the gastric mucosa against ischemia-reperfusion injury,\(^154\) which may be due to protection of mucosal microvessels. Other substances may play a role in mediating the protective effect of EGF, including sulfhydryls and somatostatin.\(^150,152,155\)

EGF has also been found to affect the thickness and chemical composition of the gastric mucus layer.\(^156\) Removal of salivary glands has been shown to cause a 35% decrease in the thickness of the oral and gastric mucus layer, and a 40% reduction in mucin content. Decreased levels of fatty acids, carbohydrates, and lipids in the mucus were observed.\(^156\) Supplementation of EGF directly into the stomach resulted in nearly complete restoration of the normal gastric mucus layer thickness and composition, whereas the normal oral mucus coat was not restored. This would suggest that maintenance of the normal mucus coat requires direct contact of salivary EGF with the epithelial surfaces.

**Acceleration of Gastric Ulcer Healing**

EGF has clearly been shown to play a role in ulcer
healing. Proposed mechanisms include mucosal regeneration, proliferation of fibroblasts and epithelial cells, neovascularization, and collagen synthesis. Several studies have reported that after removal of salivary glands in rats, the healing rate of gastric and duodenal ulcers was delayed, and DNA and RNA content was decreased in the gastric and duodenal mucosa. Administration of EGF either orally or subcutaneously completely reversed the delay in ulcer healing in sialoadenectomized rats. Oral EGF given to rats with intact salivary glands actually accelerated the healing of gastroduodenal ulcers with no influence on gastric acid secretion. This would suggest that the salivary glands and EGF play an important role in ulcer healing in rats.

In humans, decreased levels of epidermal growth factor have been found in saliva of patients with gastroduodenal ulcers and decreased concentrations in gastric juice. Whether this is related to a cause or effect of ulceration is not known.

In addition to stimulating cellular proliferation via DNA synthesis for ulcer healing, EGF promotes migration of epithelial cells, which can cover the ulcer faster than can be accounted for by mitosis. When given orally to rats with gastric and duodenal ulcers, EGF was found to bind minimally to the intact mucosa, but binding to the surface of ulcers and
small mucosal defects was pronounced. Sucralfate, an oral drug that enhances healing of gastroduodenal ulcers has been found to bind EGF in the acid pH of the stomach, and it accumulated the growth factor specifically in areas of ulceration.

Oral EGF has been reported to accelerate ulcer healing to the same extent as the histamine type 2 receptor antagonist cimetidine. Combined administration of cimetidine and EGF further increased healing of gastric ulcers, and was found to be more effective than both substances administered individually. A recent study reported that under normal acidic conditions of the stomach, gastric acid and pepsin digested the majority of the 53 amino acid EGF to primarily a 49 amino acid product within only five minutes. It was determined that the 49 amino acid EGF had only 25% of the biologic activity of the intact 53 amino acid molecule. Ulcer patients being treated with the proton pump inhibitor omeprazole had higher gastric pH values, and in those patients, the predominant gastric form of EGF was EGF$_{153}$, which possesses full biologic activity. This essentially resulted in a 3-4 fold increase in EGF concentration in the stomach, and it is an intriguing possibility that the increase in biologic activity of EGF could contribute to the healing environment.
Increased expression of EGF in the gastric mucosa has been reported in cases of gastritis\textsuperscript{167} and gastric ulcers.\textsuperscript{143,168} Immunohistochemical staining of biopsies from humans with Grade I to III gastritis showed increased EGF expression in the inflamed mucosa, with 94% of samples having immunohistochemically detectable EGF compared to 61% of normal tissue. Rats with gastric ulcers induced by acetic acid have also been found to have increased EGF immunoreactivity in the gastric mucosa.\textsuperscript{143,168} One day following exposure to acetic acid, there was an increase in the number of gland cells expressing EGF, with the highest frequency in the glands just outside the ulcer margin.\textsuperscript{168} Maximal EGF immunoreactivity was observed 3 days after ulcer induction, and was still greater than controls after 21 days.

Likewise, increased EGF receptor expression has also been reported during gastric ulcer healing.\textsuperscript{12,143} Following acetic acid induction of ulcers in rats, there was a 75-fold increase in EGF receptor density in the ulcer margin compared to controls.\textsuperscript{12} The increase was seen 3 days after ulceration, and persisted up to 25 days. A significant increase in EGF receptor expression was also found 10 millimeters distant from the ulcers. In another report, immunostaining for EGF receptor was increased with gastric ulceration, reaching a maximum at three days following ulcer induction.\textsuperscript{143} Augmented receptor
staining was still present 3 weeks later. This study found the highest frequency of cells expressing EGF receptor 1-4 millimeters from the ulcer margin, and staining then decreased with greater distance from the ulcer.

These studies have concluded that the increased EGF receptor expression in ulcerated gastric mucosa supports an important role for EGF or TGF-alpha in gastric ulcer healing.\textsuperscript{12,143} The increased receptor density distant from the ulcer suggests that cellular proliferation occurs over a widespread area, not exclusively at the ulcer margin. A cell kinetic study utilizing incorporation of H-thymidine confirmed that increased proliferation of cells was found in a region of mucosa more than 600 microns away from the ulcer.\textsuperscript{89} In addition to stimulation of cell proliferation, it was suggested that local release of EGF-like substances may serve to reduce gastric acidity and promote ulcer healing.\textsuperscript{143}

EGF receptor staining is reported to be increased in inflamed stratified squamous epithelium of the esophagus.\textsuperscript{147} There was a significant difference in the area of cells stained for EGF receptor between human patients with esophagitis compared to normal (43.1\% versus 29.5\%). Staining increases were noted in the basal as well as parabasal layers of the epithelium. Contrary to the EGF observed in the gastric mucosa of normal
patients,\textsuperscript{167} EGF staining was not detected within the squamous epithelial cells of the normal or inflamed esophagus.\textsuperscript{148} EGF was only seen in the lamina prpropria capillary endothelium immediately adjacent to basal cells of the papillae. EGF-positive capillaries were significantly decreased in inflamed mucosa of the esophagus, and it was speculated that endothelial cell stores of EGF were depleted due to demand of EGF for mitogenesis or that decreased production of EGF could contribute to the development of esophagitis.\textsuperscript{148}

**TRANSFORMING GROWTH FACTOR ALPHA (TGF-alpha)**

Transforming growth factors are peptides which were first detected in 1978 in cells transformed \textit{in vitro} by sarcoma viruses.\textsuperscript{1} Subsequently, two distinct proteins were identified, which are now known as transforming growth factor alpha and beta. Transforming growth factors are produced by normal cells, as well as by neoplastic cells, and are present in almost all human benign and malignant neoplasms.\textsuperscript{1} TGF-alpha had also been observed in embryonic cells, and it was hypothesized that TGF-alpha was an embryonic growth factor inappropriately expressed in neoplasia.\textsuperscript{92} More recently, it has become clear that TGF-alpha expression is not restricted to the embryonic and neoplastic state.\textsuperscript{169}
Cellular Distribution

TGF-alpha has a widespread distribution in normal cells and tissues. It is produced by human keratinocytes, macrophages and eosinophils, mammary epithelium, the anterior pituitary, and gastrointestinal tissue.\textsuperscript{1,92,169} In contrast to EGF, significant concentrations of TGF-alpha and TGF-alpha messenger RNA have been detected in the self-renewing epithelia of the gastrointestinal tract,\textsuperscript{102,169} which reflects true local synthesis of the growth factor rather than transport from another site.\textsuperscript{170} TGF-alpha messenger RNA has been identified in the human stomach, small intestine, and colon.\textsuperscript{92,164} TGF is expressed in the cytoplasm of human esophageal squamous epithelium,\textsuperscript{103} and similarly, TGF-alpha and its messenger RNA are also present throughout the stratified epidermis of the skin.\textsuperscript{102}

Transforming Growth Factor-alpha Structure

TGF-alpha is a functional and structural analog of EGF,\textsuperscript{1} and it shares the same receptor as epidermal growth factor. TGF-beta does not compete for the EGF receptor.\textsuperscript{1} Human TGF-alpha has a 160 amino acid transmembrane precursor protein (extracellular, transmembrane, and cytoplasmic domains) that contains the mature 50 amino acid growth factor within its extracellular domain.\textsuperscript{92} The precursor undergoes external proteolytic cleavage, and several TGF-alpha species are
released. One of several proteolytic steps is facilitated by valine located in the molecule’s cytoplasmic tail, allowing intracellular control of TGF-alpha’s release from its precursor. There is no known precursor biologic activity, although there is speculation that the unprocessed, membrane-bound TGF-alpha could be available for cell-to cell signaling processes by interaction with the EGF receptor.

The mature TGF-alpha shares 35% structural homology with the EGF molecule, and the three disulfide bridges located between cysteine residues that are found in EGF are preserved in the structure of TGF-alpha. Both TGF-alpha and EGF bind with equal affinity to the EGF receptor, both activate the receptor protein kinase, and both are capable of inducing receptor down-regulation. It has been suggested that these two peptides may bind differently to the same receptor, and that the intracellular processing and degradation of the receptor complexes may be different as well. No other receptor besides EGFr has been found that binds TGF-alpha.
FIGURE E: Structure of transforming growth factor-alpha
(From Martinez: Peptide Hormones as Prohormones, 1989)
FIGURE F: Structure of transforming growth factor-alpha precursor

(From Martinez: Peptide hormones as prohormones, 1989)
Physiologic Effects of Transforming Growth Factor-alpha

TGF-alpha has a similar spectrum of activity as EGF, although future research will likely show some differences in function. TGF-alpha is generally considered more potent than EGF in a variety of biological systems,\textsuperscript{1} and may have a longer duration of action.\textsuperscript{103} TGF-alpha is a mitogen for epithelial cells, and mediates cell migration.\textsuperscript{169} It is likely the major physiologic stimulus for keratinocytes,\textsuperscript{102} and is thought to stimulate growth of these cells by an autocrine mechanism.\textsuperscript{1} In human skin, TGF-alpha produced by keratinocytes may induce skin angiogenesis by a paracrine mechanism.\textsuperscript{1} Exogenous EGF or TGF-alpha enhances production of TGF-alpha by the keratinocyte, and may amplify wound healing following release of EGF-like peptides by platelets.\textsuperscript{102}

In the gastrointestinal tract, TGF-alpha is produced within the normal gastric mucosa, whereas no EGF production has been identified.\textsuperscript{164} In the stomach, TGF-alpha has been reported to inhibit gastric acid secretion, stimulate proliferation of gastric mucosa, and enhance gastric mucin production.\textsuperscript{92}

Parenteral TGF-alpha was reported to be twice as potent as EGF in the inhibition of gastric acid secretion.\textsuperscript{3} TGF-alpha messenger RNA and TGF-alpha expression were pronounced in guinea pig parietal cells, whereas EGF or EGF mRNA were not.\textsuperscript{164}
raising the possibility that locally-produced TGF-alpha may regulate acid secretion by the parietal cell via autocrine and paracrine mechanisms.\textsuperscript{138,164} Coffey\textsuperscript{169} theorized that TGF-alpha could suppress basal gastric acid production by binding to the EGF receptor in tubulovesicles of quiescent parietal cells; when parietal cells are activated and fuse to the canalculus, acid production by the H\textsuperscript{+}, K\textsuperscript{+} ATPase would dissociate TGF-alpha from its receptor, releasing it into the stomach lumen and removing the acid inhibition.

Similar to EGF, TGF-alpha also protects the gastric mucosa against ethanol damage when administered parenterally 30 minutes prior to ethanol exposure.\textsuperscript{3} TGF-alpha applied intragastrically failed to provide protection against mucosal damage.\textsuperscript{3} Parenteral TGF-alpha has been reported to increase levels of gastric mucin in a time and dose-dependent fashion, and that mucin production corresponded to the timing of TGF-induced mucosal protection.\textsuperscript{169}

Interestingly, the highest levels of TGF-alpha mRNA in the small intestine have been found in the terminally differentiated villus tip cells, as opposed to the crypts.\textsuperscript{92} Likewise, localization of TGF-alpha in the small intestine and colon was to the upper one-third of the crypt,\textsuperscript{170} suggesting that its location in the fully differentiated compartment may
indicate a primary role for TGF-alpha other than as a mitogen. Because of the widespread production of TGF-alpha and the limited known EGF sites of origin, it has been theorized that in vivo, TGF-alpha is most likely the major ligand for the EGF receptor rather than EGF.\textsuperscript{169,170}
EPIDERMAL GROWTH FACTOR RECEPTOR IN EQUINE GASTRIC STRATIFIED SQUAMOUS EPITHELIAL MUCOSA: EFFECT OF PROGRESSIVE ULCERATION ON RECEPTOR DENSITY

An ulcer induction protocol recently developed in horses closely parallels physiologic mechanisms of gastric ulceration in that species by exposing the stomach to periods of excessive gastric acidity. The objectives for the study reported here, utilizing this protocol to induce gastric ulcers in the squamous epithelial mucosa of the horse, were to document the presence of epidermal growth factor (EGF) receptor in equine gastric mucosa, quantitate the receptor density in normal mucosa, and to quantitate the receptor density at 2 stages of gastric ulceration. Studies in the glandular mucosa of the rat stomach reported that the greatest increases in EGF receptor density occurred at the ulcer margin and within several millimeters of the margin. We sought to determine if there were peptic injury-associated increases of EGF receptor density in the equine gastric squamous epithelium, and if so, where those increases occurred in relation to gastric lesions.

MATERIALS AND METHODS

Animals

Fifteen horses, aged 3-20 years, were used for the study. Ten horses that were purchased or donated underwent the experimental protocol, and five additional donated horses were
used as normal controls. Horses that underwent the ulcer induction protocol were dewormed and maintained on pasture for a minimum of two weeks prior to the study. Following the acclimation period, gastroscopy was performed to determine the presence of any pre-existing gastric lesions. Horses began the ulcer induction protocol only if the stomach appeared normal on endoscopic examination. The stomachs of the five control horses were determined to be normal by gastroscopy prior to euthanasia or at post-mortem examination.

Protocol for induction of gastric ulcers

The horses were kept in 12' x 12' box stalls with free choice water at all times. There were three groups of five horses each; Group 1 was a normal control, and Groups 2 and 3 developed gastric lesions. Gastric ulcers were induced by alternately feeding and withholding free choice timothy grass hay for 24 hour periods. Group 2 was fed for one 24-hour period, and hay was withheld for two 24-hour periods. Group 3 was fed for four 24-hour periods and hay was withheld for four 24-hour periods.

<table>
<thead>
<tr>
<th>Study Group (5 horses each)</th>
<th>Off Feed</th>
<th>Total Protocol Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control)</td>
<td>0 hours</td>
<td>0 hours</td>
</tr>
<tr>
<td>Group 2</td>
<td>48 hours</td>
<td>72 hours</td>
</tr>
<tr>
<td>Group 3</td>
<td>96 hours</td>
<td>192 hours</td>
</tr>
</tbody>
</table>
Gastroscopy was performed on Groups 2 and 3 after 48 hours of feed deprivation, and on Group 3 after 96 hours of feed deprivation. Horses were sedated with 0.5 mg/kg of xylazine intravenously, and a 2 meter video endoscope (Welch Allyn, Skaneateles Falls NY) was used to examine the stomach. All of the squamous epithelial mucosa of the stomach was examined, as well as (approximately 40-50%) the glandular mucosa that was visible above the gastric secretions. Endoscopic images were captured and digitized on a Compaq portable computer (Compaq Computer Corp, Houston TX) with a Targa-16 (Truevision) board, and then saved on an optical disk.

**Tissue collection**
All horses were humanely euthanatized with an overdose of barbiturate. Horses in Group 2 were euthanatized after Day 3 of the protocol (48 hours off feed), and horses in Group 3 after Day 8 (96 hours off feed). Stomachs were collected intact by severing the distal esophagus and proximal duodenum. All ingesta was rinsed from the lumen by flushing water into the stomach through the duodenum, then draining the water from the stomach. Approximately four liters of 10% buffered formalin were infused into each stomach, and the esophagus and duodenum were tied off. The stomachs were placed into buckets with 8 liters of buffered formalin and allowed to fix in situ for 24 hours. Full-thickness sections of gastric lesions and
normal tissue were obtained and placed in 10% buffered formalin. Tissue sections were then embedded in paraffin blocks.

**Immunohistochemistry**

An avidin-biotin immunoperoxidase technique was used to stain the gastric tissues for epidermal growth factor receptor. Tissue sections 5 microns thick were mounted on charged slides, then deparaffinized and rehydrated by passage through xylene and absolute ethanol. Endogenous peroxidase activity was blocked with 30% hydrogen peroxide in methanol for 10 minutes. Nagarse Protease 0.1% Type XXVII (Sigma Chemical Co.) was applied to tissues for 20 minutes at 37 degrees C for enzyme digestion of aldehyde linkages that mask tissue antigen. Sections were then incubated with undiluted horse serum to block non-specific protein binding sites. A primary, mouse monoclonal epidermal growth factor receptor antibody (Triton Laboratories, Alameda CA) diluted 1:150 was applied to tissues for 60 minutes at room temperature. Sections were then incubated for 30 minutes with a biotinylated, anti-mouse IgG secondary antibody, followed by 30 minutes with an avidin-biotinylated horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame CA). Slides were developed with the chromagen 3,3'-diaminobenzidine (DAB) for 5 minutes. A chromagen enhancing solution (Vector
Laboratories) was then applied for 5-10 seconds. Tissue sections were counterstained with Gill’s II hematoxylin, decolorized in 4% acetic acid, and allowed to blue in lithium carbonate. The slides were then dehydrated with absolute ethanol, graded alcohols and xylene.

Histologic image selection and image analysis

Stained microscope slides were photographed using the 20X objective (Olympus photomicroscope, Olympus Optical Co, Tokyo JP), and the 35-mm slides were then scanned (Coolscan, Nikon Inc, Melville NY) into a computer (MacIntosh Quadra 950, Apple Computer Inc, Cupertino CA).

Tissues were examined from the fundus, the margo plicatus on the right side of the stomach, and the lesser curvature. Lesions were then characterized as ulcers or erosions. An ulcer was defined as gastric tissue that had loss of all epithelial layers with exposure of the lamina propria to the gastric lumen. An erosion was defined as having incomplete epithelial loss, with intact epithelium still present over the lamina propria.

If no lesions were present in the area of the stomach being examined (fundus, margo plicatus right, lesser curvature), one full-thickness representative image of that area was
evaluated. If an ulcer or erosion was present, three images of the lesion were evaluated. These areas included: 1) ulcer/erosion margin, 2) erosion bed (omit for ulcer), and 3) tissue 10-14 mm distant from the ulcer/erosion.

Measurements relating to EGF receptor were made at four different vertical intervals within the epithelium, using a 2,500 square-micron sample block (a square with sides of 50 microns in length) at each location. Each sample block was calibrated (pixels per square micron) with a 2,500 square-micron square image digitized from a hemocytometer grid. Measurements were taken 1) at the basal layer of the squamous epithelium (Basal), 2) above the basal layer where cells are polyhedral in shape (Middle 1), 3) at the transition between polyhedral cells and flattened, spindle-shaped cells (Middle 2), 4) just below cornified cells closest to the lumen (Upper). See Figure 1 on the following page.

With images from erosion beds, measurements were taken at two intervals of the epithelium. A 2,500 square-micron area was measured 1) at the basal layer (Basal), and 2) half-way between the basal layer and the luminal surface of the erosion bed (Middle). This value was subsequently compared to the Middle 1 measurements from the ulcer/erosion margin and distant from the lesion.
A computerized, image analysis system (Horsepath, Loats Associates Inc, Westminster MD) that utilized image separations based upon hue (H), saturation (S), and intensity (I) was used to quantitate epidermal growth factor receptor in the histologic tissue sections. Image separation using the HSI system allowed specific selection of EGF receptor staining and elimination of all other image components. Hue was used to define the color of the region of interest (EGF receptor),
with hues being the colors within the visible spectrum. The intensity separation was a black and white version of the color image, and corresponded to varying image brightness levels. The saturation separation was not used in this analysis.

EGF receptor stain color was selected on the hue separation, and all pixels without receptor stain color were excluded. The selected pixels were applied to the intensity separation, resulting in pixels with receptor stain only, as well as the intensity of those pixels. The software measured the mean intensity value of pixels in each 2,500 square-micron sample area that had been selected for receptor stain. Measurements calculated from each 2,500 square-micron sample were 1) EGFr area, 2) percent EGFr area, and 3) mean EGFr intensity value. EGFr area was defined as the number of square microns within the 2,500 square-micron sample block that contained EGFr staining, and the percent EGFr area was calculated by dividing EGFr area in square microns by 2,500. EGFr intensity was an estimation of the density of EGFr receptor present. Intensity values were based upon 256 gray-scale levels from black (0) to white (256), with a numerical value between 0-256 assigned to each pixel. A mean intensity value was then calculated for each 2,500 square-micron sample block, with lower values corresponding to darker receptor staining. Subsequently, all
references to receptor intensity measurements will refer to intensity, such that low mean intensity values will correspond to high EGFr intensity.

EGFr area, percent EGFr area, and mean EGFr intensity values were also measured around selected epithelial capillaries. Capillaries close to the basal epithelial layer were selected, as well as those present in the superficial layers closer to the gastric lumen. Measurements were taken 1) within an elliptical ring which incorporated one epithelial cell layer around the capillary circumference (Region A), and 2) within a second concentric elliptical ring around Region A that was 20% larger (Region B). Since all capillaries were sized differently, EGFr area was variable, therefore comparisons relating to EGFr area were made using percent EGFr area. See Figure 2 on following page.

Lastly, mitotic figures were recorded per 100 basal cells counted in the lesion margin, bed, and distant from the lesion in ulcerated/eroded epithelium (Groups 2 and 3), as well as in the normal-appearing mucosa (Group 1).
FIGURE 2

Statistical analysis

The mean and standard deviation values were obtained for EGFr area and the mean EGFr intensity value in the gastric squamous epithelial tissue from all 3 groups. Comparisons of the mean EGFr area and mean EGFr intensity values were then made by
stomach location (fundus, margo plicatus right, lesser curvature) using a one-way analysis of variance (ANOVA) 1) **between groups** (Group 1 vs Group 2, Group 1 vs Group 3, and Group 2 vs Group 3) and 2) **by lesion site within a group** (ulcer/erosion margin, erosion bed, distant from the lesion in Groups 2 and 3). Experiment-wise comparison results were considered statistically significant at p ≤ 0.05.

The mean and standard deviation values were also obtained for percent EGFr area and mean EGFr intensity value adjacent to epithelial capillaries. Comparisons of the mean percent EGFr area and mean EGFr intensity value were made using a one-way ANOVA, 1) **within groups** (Groups 1, 2, and 3) between Region A and Region B, and 2) **between groups** (Regions A and B of Control Group 1 compared to Regions A and B of Groups 2 and 3).

A one-way ANOVA was used to compare the mean number of mitotic figures per 100 basal cells by stomach location (fundus, margo plicatus right, lesser curvature) 1) **between groups with lesions and the Control group** (Group 1 vs Group 2, and Group 1 vs Group 3).
RESULTS

**Gastric squamous lesions** (Groups 2 and 3)

All horses that underwent the experimental protocol developed gastric squamous lesions. Gastric ulceration or erosion was present primarily in the squamous epithelium along the margo plicatus on the right side of the stomach and along the lesser curvature. Only one horse (Group 2) developed lesions in the squamous fundus of the stomach. None of the horses in either experimental group had lesions in the glandular mucosa.

Endoscopically, gastric lesions ranged in appearance from mild, superficial erosions to diffuse linear ulcers/erosions, and deep bleeding ulcers. (Table 1) Some of the lesions in Group 2 horses had raised margins consistent with epithelial proliferation. Most lesions seen in Group 3 had raised, thickened margins, and margins appeared to have contracted since the examination following 48 hours of feed deprivation.

Histologically, ulcers from both Groups 2 and 3 had epithelial loss, inflammatory cell infiltration, and proliferation of capillaries within the lamina propria. Capillary proliferation in the lamina propria was prominent. Group 3 horses had marked mucosal proliferation at the ulcer/erosion margins, with increased thickness of all epithelial layers. The epithelium at the margin had well-developed epithelial projections.
TABLE 1: ENDOSCOPIC APPEARANCE OF GASTRIC LESIONS INDUCED BY EXPERIMENTAL PROTOCOL (Margo Plicatus Right and Lesser Curvature)

<table>
<thead>
<tr>
<th>HORSES</th>
<th>GASTROSCOPY 48 Hrs</th>
<th>GASTROSCOPY 96 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horse 1</td>
<td>Mild/moderate, linear ulceration with keratinization at margins</td>
<td></td>
</tr>
<tr>
<td>Horse 2</td>
<td>Deep, coalesced ulcers at margo plicatus</td>
<td></td>
</tr>
<tr>
<td>Horse 3</td>
<td>Severe, bleeding, linear ulceration. some with raised margins. Large areas of epithelial loss</td>
<td></td>
</tr>
<tr>
<td>Horse 4</td>
<td>Multifocal, deep ulceration, hyperkeratosis</td>
<td></td>
</tr>
<tr>
<td>Horse 5</td>
<td>Moderate, linear ulceration covered with necrotic debris, some with thickened margins</td>
<td></td>
</tr>
<tr>
<td>GROUP 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horse 6</td>
<td>Moderate, diffuse ulceration at lesser curvature, some with raised margins, hyperkeratosis</td>
<td>Diffuse, deep ulcers with raised, thickened margins, hyperkeratosis</td>
</tr>
<tr>
<td>Horse 7</td>
<td>Moderate, linear erosions, hyperemia</td>
<td>Moderate, contracted linear erosions, ulcers with raised, thickened margins</td>
</tr>
<tr>
<td>Horse 8</td>
<td>Mild, linear erosions, hyperkeratosis</td>
<td>Moderate, linear erosions with raised, thickened margins, hyperkeratosis</td>
</tr>
<tr>
<td>Horse 9</td>
<td>Moderate, diffuse ulceration covered with necrotic debris, some with thickened margins</td>
<td>Diffuse, contracted ulcers with raised, thickened margins</td>
</tr>
<tr>
<td>Horse 10</td>
<td>Hyperkeratosis, no lesions</td>
<td>Few, small erosions at lesser curvature, hyperkeratosis</td>
</tr>
</tbody>
</table>

Tissue distribution of EGF receptor

There was positive staining for EGF receptor (EGFR) in all gastric squamous epithelia. Staining for EGFR was localized primarily to epithelial cell membranes, with occasional cytoplasmic staining in basal or parabasal epithelial cells.
EGFr staining was absent in the lamina propria, submucosa and muscular layers of the epithelium. Figure 3 shows positive staining for EGFr in a normal squamous epithelial tissue section at the margo plicatus taken from the right side of the stomach.

In all groups, mean EGFr area and intensity were greatest (p<0.05) in the basal layer, and both progressively decreased toward the luminal surface (superficial epithelial layers). Mean EGFr area and intensity value measurements by tissue from all 3 groups are shown in Tables 2 to 6.

**TABLE 2: EGFR RECEPTOR AREA AND MEAN INTENSITY VALUE (Fundus):**

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>GROUP 1 (Normal)</th>
<th>GROUP 2 (48 Hrs)</th>
<th>GROUP 3 (96 Hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean EGFr Area</td>
<td>Mean EGFr Area</td>
<td>Mean EGFr Area</td>
</tr>
<tr>
<td>FUNDUS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1676.0 (67%) ± 136.5^a</td>
<td>1499.3 (60%) ± 104.1^b</td>
<td>1493.2 (60%) ± 91.4^c</td>
</tr>
<tr>
<td>Middle 1</td>
<td>1238.0 (50%) ± 54.3^d</td>
<td>939.0 (39%) ± 188.3^b</td>
<td>1686.0 (43%) ± 233.0^c</td>
</tr>
<tr>
<td>Middle 2</td>
<td>679.8 (27%) ± 38.4^d</td>
<td>436.0 (17%) ± 230.0^b</td>
<td>681.3 (27%) ± 144.4^c</td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
<td>No receptor present</td>
<td>No receptor present</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FUNDUS</th>
<th>Mean EGFr Intensity Value</th>
<th>Mean EGFr Intensity Value</th>
<th>Mean EGFr Intensity Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>96.50 ± 13.28^de</td>
<td>97.5 ± 18.27^f</td>
<td>96.00 ± 12.91^g</td>
</tr>
<tr>
<td>Middle 1</td>
<td>126.50 ± 7.51^e</td>
<td>124.7 ± 26.70</td>
<td>133.25 ± 12.34</td>
</tr>
<tr>
<td>Middle 2</td>
<td>136.75 ± 10.05^d</td>
<td>136.2 ± 25.60^f</td>
<td>152.00 ± 16.87^g</td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
<td>No receptor present</td>
<td>No receptor present</td>
</tr>
</tbody>
</table>

EGFr area measured in square microns within a 2500 square micron sample.
EGFr intensity value measured on a scale of 0 (black) to 256 (white). Lower mean EGFr intensity value indicates darker EGFr staining.
Superscript (a-g): Within vertical groups, values with the same superscript are significantly (p < 0.05) different.
TABLE 3: EGF RECEPTOR AREA (Margo Plicatus Right): Mean and Standard Deviation Values

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>GROUP 1 (Normal)</th>
<th>GROUP 2 (48 Hrs)</th>
<th>GROUP 3 (96 Hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean EGF&lt;sub&gt;r&lt;/sub&gt; Area</td>
<td>Mean EGF&lt;sub&gt;r&lt;/sub&gt; Area</td>
<td>Mean EGF&lt;sub&gt;r&lt;/sub&gt; Area</td>
</tr>
<tr>
<td>Margo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plicatus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (n=5)</td>
<td>1678.5 (67%) ± 259.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1503 (60%) ± 248.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1652.5 (66%) ± 119.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Basal</td>
<td>1152.9 (46%) ± 148.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1168 (47%) ± 233.0</td>
<td>1220.5 (48%) ± 55.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Middle 1</td>
<td>629.2 (25%) ± 76.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>839 (34%) ± 219.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>718.0 (29%) ± 49.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Middle 2</td>
<td>No receptor present</td>
<td>No receptor present</td>
<td>No receptor present</td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
<td>No receptor present</td>
<td>No receptor present</td>
</tr>
<tr>
<td>Margin (n=4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1503 (60%) ± 248.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1652.5 (66%) ± 119.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 1</td>
<td>1168 (47%) ± 233.0</td>
<td>1220.5 (48%) ± 55.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 2</td>
<td>839 (34%) ± 219.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>718.0 (29%) ± 49.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
<td>No receptor present</td>
<td>No receptor present</td>
</tr>
<tr>
<td>Erosion Bed (n=3)</td>
<td>1251.7 (50%) ± 145.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1433.0 (57%) ± 212.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>976.0 (39%) ± 169.0</td>
<td>1145.0 (46%) ± 47.8&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle</td>
<td>976.0 (39%) ± 169.0</td>
<td>1145.0 (46%) ± 47.8&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Distant (n=4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1786.0 (71%) ± 223.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1860.0 (74%) ± 161.3&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 1</td>
<td>1290.0 (52%) ± 169.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1186.0 (47%) ± 209.0&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 2</td>
<td>960.5 (38%) ± 92.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>646.5 (26%) ± 164.9&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
<td>No receptor present</td>
<td>No receptor present</td>
</tr>
</tbody>
</table>

EGFr area measured in square microns within a 2500 square micron sample.
† Significantly (p < 0.05) different from Group 1 (Normal).
‡ Significantly (p < 0.05) different from Group 2 (48 Hours).
Superscript (a-f): Within vertical groups, values with the same superscript are significantly (p < 0.05) different.
TABLE 4: EGF RECEPTOR MEAN INTENSITY VALUE (Margo Plicatus Right): Mean and Standard Deviation Values

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>GROUP 1 (Normal)</th>
<th>GROUP 2 (48 Hrs)</th>
<th>GROUP 3 (96 Hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MARGO PLICATUS</td>
<td>Mean EGF&lt;sub&gt;r&lt;/sub&gt; Intensity Value</td>
<td>Mean EGF&lt;sub&gt;r&lt;/sub&gt; Intensity Value</td>
<td>Mean EGF&lt;sub&gt;r&lt;/sub&gt; Intensity Value</td>
</tr>
<tr>
<td>Normal (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>95.75 ± 8.15&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle 1</td>
<td>132.75 ± 10.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle 2</td>
<td>139.50 ± 10.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Margin (n=4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td></td>
<td>104.80 ± 14.24</td>
<td>92.50 ± 13.10&lt;sup&gt;c&lt;/sup&gt;&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Middle 1</td>
<td>130.25 ± 19.97</td>
<td>126.50 ± 8.06&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 2</td>
<td>130.00 ± 18.81</td>
<td>137.75 ± 7.04&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
<td>No receptor present</td>
<td></td>
</tr>
<tr>
<td>Erosion Bed</td>
<td>(n=3)</td>
<td>(n=4)</td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>120.33 ± 10.79&lt;sup&gt;†&lt;/sup&gt;</td>
<td>111.23 ± 12.82&lt;sup&gt;†&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle</td>
<td>130.67 ± 16.62</td>
<td>124.25 ± 10.24</td>
<td></td>
</tr>
<tr>
<td>Distant (n=4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>89.75 ± 19.05&lt;sup&gt;e&lt;/sup&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>86.75 ± 8.96&lt;sup&gt;g&lt;/sup&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 1</td>
<td>141.00 ± 9.42&lt;sup&gt;g&lt;/sup&gt;</td>
<td>127.75 ± 9.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 2</td>
<td>157.50 ± 13.38&lt;sup&gt;g&lt;/sup&gt;&lt;sup&gt;†&lt;/sup&gt;</td>
<td>138.75 ± 16.17&lt;sup&gt;g&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
<td>No receptor present</td>
<td></td>
</tr>
</tbody>
</table>

EGFr intensity value measured on a scale of 0 (black) to 256 (white). Lower mean EGF<sub>r</sub> intensity value indicates darker EGF<sub>r</sub> staining.

† Significantly (p < 0.05) different from Group 1 (Normal).

§ Significantly (p < 0.05) different from Group 2 (48 Hours).

Superscript (a-h): Within vertical groups, values with the same superscript are significantly (p < 0.05) different.
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>GROUP 1 (Normal)</th>
<th>GROUP 2 (48 Hrs)</th>
<th>GROUP 3 (96 Hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LESHER CURVATURE</strong></td>
<td><strong>Mean EGFr Area</strong></td>
<td><strong>Mean EGFr Area</strong></td>
<td><strong>Mean EGFr Area</strong></td>
</tr>
<tr>
<td>Normal (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1560.6 (62%) ± 162.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle 1</td>
<td>1136.0 (45%) ± 71.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle 2</td>
<td>757.0 (30%) ± 156.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Margin (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1542.0 (62%) ± 223.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1850.0 (74%) ± 197.6&lt;sup&gt;++&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 1</td>
<td>1024.4 (41%) ± 129.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1324.2 (53%) ± 153.3&lt;sup&gt;++&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 2</td>
<td>708.8 (28%) ± 120.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>677.2 (27%) ± 67.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
<td>No receptor present</td>
<td></td>
</tr>
<tr>
<td>Erosion Bed (n=4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1298.0 (52%) ± 278.0</td>
<td></td>
<td>1562.6 (63%) ± 153.5&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Middle</td>
<td>1035.0 (41%) ± 33.0&lt;sup&gt;†&lt;/sup&gt;</td>
<td>1277.4 (51%) ± 108.6&lt;sup&gt;++&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Distant (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1844.2 (74%) ± 214.7&lt;sup&gt;†&lt;/sup&gt;</td>
<td>1807.8 (72%) ± 183.0&lt;sup&gt;†&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 1</td>
<td>1172.6 (47%) ± 147.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1233.8 (49%) ± 85.5&lt;sup&gt;†&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 2</td>
<td>689.8 (28%) ± 146.6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>669.8 (27%) ± 59.7&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
<td>No receptor present</td>
<td></td>
</tr>
</tbody>
</table>

EGF<sub>r</sub> area measured in square microns within a 2500 square micron sample.

† Significantly (p < 0.05) different from Group 1 (Normal).

++ Significantly (p < 0.05) different from Group 2 (48 Hours).

Superscript (a-f): Within vertical groups, values with the same superscript are significantly (p < 0.05) different.
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>GROUP 1 (Normal)</th>
<th>GROUP 2 (48 Hrs)</th>
<th>GROUP 3 (96 Hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesser Curvature</td>
<td>Mean EGFr</td>
<td>Mean EGFr</td>
<td>Mean EGFr</td>
</tr>
<tr>
<td></td>
<td>Intensity Value</td>
<td>Intensity Value</td>
<td>Intensity Value</td>
</tr>
<tr>
<td>Normal (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>93.60 ± 6.47&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle 1</td>
<td>120.00 ± 8.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle 2</td>
<td>126.00 ± 9.59&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
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<td></td>
</tr>
<tr>
<td>Margin (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>94.40 ± 16.98&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>91.20 ± 13.39&lt;sup&gt;ah&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 1</td>
<td>138.20 ± 13.99&lt;sup&gt;†&lt;/sup&gt;</td>
<td>138.00 ± 15.46&lt;sup&gt;†&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 2</td>
<td>156.40 ± 14.89&lt;sup&gt;†&lt;/sup&gt;</td>
<td>150.60 ± 14.33&lt;sup&gt;†&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
<td>No receptor present</td>
<td></td>
</tr>
<tr>
<td>Erosion Bed (n=4)</td>
<td>110.50 ± 30.20</td>
<td>(n=5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>143.25 ± 5.91†</td>
<td>108.60 ± 14.15&lt;sup&gt;†&lt;/sup&gt;</td>
<td>131.00 ± 11.47&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>Distant (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>79.60 ± 16.41&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>93.00 ± 15.30&lt;sup&gt;†&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 1</td>
<td>145.00 ± 16.85&lt;sup&gt;†&lt;/sup&gt;</td>
<td>132.20 ± 9.20&lt;sup&gt;†&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Middle 2</td>
<td>158.20 ± 10.78&lt;sup&gt;†&lt;/sup&gt;</td>
<td>153.60 ± 17.76&lt;sup&gt;†&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>No receptor present</td>
<td>No receptor present</td>
<td></td>
</tr>
</tbody>
</table>

EGF<sub>r</sub> intensity value measured on a scale of 0 (black) to 256 (white). Lower mean EGF<sub>r</sub> intensity value indicates darker EGF<sub>r</sub> staining.
† Significantly (p < 0.05) different from Group 1 (Normal).
‡ Significantly (p < 0.05) different from Group 2 (48 Hours).
Superscript (a-k): Within vertical groups, values with the same superscript are significantly (p < 0.05) different.
COMPARISON: EGFr area and mean intensity values between groups

**Fundus**: There were no significant differences in EGFR area or mean intensity values between Groups 1, 2, and 3.

**Margo Plicatus Right**

*Ulcer/erosion margin*: EGFr area (Middle 2) in the ulcer/erosion margin of Group 2 was significantly greater (p=0.030) than Control Group 1, and that of Group 3 was nearly significantly greater (p=0.062) than Group 1. There were no significant differences in mean EGFr intensity values in the ulcer/erosion margins of Groups 2 and 3 when compared to Control Group 1.

*Erosion bed*: In contrast to the margin, EGFr area in the erosion bed of Group 2 was significantly less than both Groups 1 and 3 (Group 1 Basal: p=0.027; Group 3 Middle: p=0.046). The mean EGFr intensity value (Basal) in the erosion bed of Groups 2 and 3 was significantly greater (lighter EGFr staining) than Control Group 1 (Group 2: p=0.003, Group 3: p=0.027).

*Distant*: Distant from the lesions, the EGFr area (Middle 2) of Group 2 was significantly greater (p=0.001) than both Groups 1 and 3. The mean EGFr intensity value (Middle 2) of Group 2 was significantly greater (lighter EGFr staining) than Group
1 (p=0.028).

**Lesser Curvature**

*Ulcera/erosion margin:* This tissue region showed pronounced changes in Group 3. EGFr area (Basal, Middle 1) in the margin of Group 3 was significantly greater than both Groups 1 and 2 (Group 1: Basal p=0.035, Middle 1 p=0.038; Group 2: Basal p=0.050, Middle 1 p=0.010). The mean EGFr intensity value (Middle 1, Middle 2) in the margin of Groups 2 and 3 was significantly greater (lighter EGFr staining) than Control Group 1 (Group 2: Middle 1 p=0.038, Middle 2 p=0.005; Group 3: Middle 1 p=0.052, Middle 2 p=0.013).

*Erosion bed:* EGFr area in the erosion bed of Group 3 (Middle) was significantly greater than both Groups 1 and 2 (Group 1: p=0.042, Group 2: p=0.004). EGFr area in the erosion bed of Group 2 (Middle) was significantly less (p=0.036) than Control Group 1. The mean EGFr intensity value (Middle) of the erosion bed of Group 2 was significantly greater (lighter EGFr staining) than Group 1 (p=0.001), and that of Group 3 was nearly significantly greater (p=0.063) than Group 1.

*Distant:* Distant from the lesion, EGFr area (Basal) of Groups 2 and 3 was significantly greater (Group 2 p=0.046; Group 3 p=0.054) than Control Group 1. The mean EGFr intensity values
of Groups 2 and 3 were greater (lighter EGFr staining) than Group 1 (Group 2: Middle 1 p=0.018, Middle 2 p=0.001; Group 3: Middle 2 p=0.016).

COMPARISON: **EGFr staining within Groups 2 and 3 (lesion sites)**

**Margo Plicatus Right**

*Erosion margin vs erosion bed:* There were no significant differences in EGFr area or mean EGFr intensity value between the erosion margin and erosion bed within Groups 2 or 3.

*Ulcer/erosion margin vs distant:* There were no significant differences in EGFr area or mean EGFr intensity value between the ulcer/erosion margin and distant from the lesions within Groups 2 or 3.

*Erosion bed vs distant:* The EGFr area (Basal) of the erosion bed in both Groups 2 and 3 was significantly less (Group 2: p=0.016; Group 3: p=0.019) than distant from the lesions. Similarly, the EGFr intensity value (Basal) was significantly greater (lighter EGFr staining) in the erosion bed of Group 3 than distant from the lesions (p=0.020), and nearly significantly greater in the erosion bed of Group 2 (p=0.057).
Lesser Curvature

Erosion margin vs erosion bed: There was no significant difference in EGFr area between the erosion margin and erosion bed of Group 2, but EGFr area (Basal) in the margin of Group 3 was significantly greater (p=0.033) than the erosion bed of that group. There were no significant differences in the mean EGFr intensity values.

Ulcer/erosion margin vs distant: There were no differences in EGFr area or mean EGFr intensity value between the margin and distant from the lesions in Group 2 or 3.

Erosion bed vs distant: EGFr area (Basal) in the erosion bed of Groups 2 and 3 was significantly less (Group 2: p=0.013; Group 3: p=0.050) than distant from the lesions. There were no significant differences in the mean EGFr intensity value.

COMPARISON: EGFr adjacent to epithelial capillaries
Mean values for %EGFr area and mean EGFr intensity value in squamous epithelial cells surrounding capillaries (Region A and B) are shown in Table 7. EGFr area in Region A immediately adjacent to capillaries of Groups 1, 2, and 3 was significantly greater (Group 1: p=0.002; Group 2: p=0.001; Group 3: p=0.001) than Region B within those groups. Comparing between groups, the EGFr area in Region A around capillaries
of Group 3 was significantly greater (p=0.026) than Region A of the Control Group 1. No differences were found between Region B of those groups.

The EGFr intensity value in Region A and B of Group 3 was significantly greater (lighter EGFr staining) than the Control Group 1 (Region A: p=0.004; Region B: p=0.002).

**TABLE 7: MEAN %EGFr AREA AND MEAN INTENSITY VALUE: Adjacent to Epithelial Capillaries**

<table>
<thead>
<tr>
<th>% EGFr AREA</th>
<th>GROUP 1 (Normal)</th>
<th>GROUP (48 Hrs)</th>
<th>GROUP 3 (96 Hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region A (Adjacent to capillary)</td>
<td>57.60% ± 9.28a</td>
<td>65.36% ± 14.87b</td>
<td>68.37% ± 12.16c*</td>
</tr>
<tr>
<td>Region B (Adjacent to Region A)</td>
<td>35.96% ± 16.98a</td>
<td>35.84% ± 11.99b</td>
<td>26.97% ± 13.11c</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MEAN EGFr INTENSITY VALUE</th>
<th>GROUP 1 (Normal)</th>
<th>GROUP 2 (48 Hrs)</th>
<th>GROUP 3 (96 Hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region A (Adjacent to capillary)</td>
<td>119.2 ± 17.73</td>
<td>130.69 ± 21.78</td>
<td>140.13 ± 15.23*</td>
</tr>
<tr>
<td>Region B (Adjacent to Region A)</td>
<td>128.7 ± 16.81</td>
<td>138.46 ± 20.39</td>
<td>151.20 ± 14.90*</td>
</tr>
</tbody>
</table>

EGFr intensity value measured on a scale of 0 (black) to 256 (white). Lower mean intensity value indicates darker EGFr staining.

*Significantly (p < 0.05) different from Control Group 1.

Superscript (a-c): Within vertical groups, values with the same superscript are significantly (p < 0.05) different.

**Mitotic figures**

**Fundus:** There were no significant differences in mean number of mitotic cells per 100 basal cells between the fundus of Groups 2 and 3 and Control Group 1. (Table 8)
Margo Plicatus Right: The mean number of mitotic figures per 100 basal cells in the ulcer/erosion margin, erosion bed, and distant from the lesions in Groups 2 and 3 were significantly greater than Control Group 1. (Group 2 margin: p=0.001, Group 3 margin: p=0.001; Group 2 bed: p=0.022, Group 3 bed: p=0.002; Group 2 distant: p=0.034, Group 3 distant: p=0.034 respectively).

Lesser Curvature: The mean number of mitotic figures per 100 basal cells in the ulcer/erosion margin, erosion bed, and distant from the lesions in Groups 2 and 3 were significantly greater than Control Group 1 (Group 2 margin: p=0.001, Group 3 margin: p=0.001; Group 2 bed: p=0.011, Group 3 bed: p=0.001; Group 2 distant: p=0.013, Group 3 distant: p=0.050).

**TABLE 8: NUMBER OF MITOTIC FIGURES PER 100 BASAL EPITHELIAL CELLS: Mean and Standard Deviation Values**

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>GROUP 1 (Normal) Mitotic figures/100 cells</th>
<th>GROUP 2 (48 Hrs) Mitotic figures/100 cells</th>
<th>GROUP 3 (96 Hrs) Mitotic figures/100 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FUNDUS</strong></td>
<td>0.75 ± 0.50</td>
<td>0.75 ± 0.50</td>
<td>0.75 ± 0.50</td>
</tr>
<tr>
<td><strong>MARGO Plicatus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Margin</td>
<td>0.75 ± 0.46</td>
<td>2.75 ± 0.50*</td>
<td>3.00 ± 0.82*</td>
</tr>
<tr>
<td>Erosion bed</td>
<td></td>
<td>1.67 ± 0.58*</td>
<td>2.50 ± 1.00*</td>
</tr>
<tr>
<td>Distant from lesion</td>
<td></td>
<td>1.50 ± 0.58*</td>
<td>1.50 ± 0.58*</td>
</tr>
<tr>
<td><strong>LESSER CURVATURE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Margin</td>
<td>1.00 ± 0.00</td>
<td>2.80 ± 0.45*</td>
<td>3.00 ± 0.00*</td>
</tr>
<tr>
<td>Erosion bed</td>
<td></td>
<td>1.75 ± 0.50*</td>
<td>2.20 ± 0.45*</td>
</tr>
<tr>
<td>Distant from lesion</td>
<td></td>
<td>2.00 ± 0.71*</td>
<td>1.80 ± 0.84*</td>
</tr>
</tbody>
</table>

*significantly (p < 0.05) greater than Control Group 1 (Normal).
Figure 3:
Epidermal growth factor receptor (EGFr) staining in normal equine gastric squamous mucosa (margo plicatus on the right side of the stomach). 20X magnification; bar in right corner= 50 microns.
Figure 4: EGFr staining in the ulcer/erosion margin from the lesser curvature after 96 hours of feed deprivation (Group 3). 20X magnification; bar in right corner=50 microns.
Figure 5: EGFr staining in the erosion bed from the margo plicatus after 96 hours of feed deprivation (Group 3). 20X magnification; bar in right corner=50 microns.
Figure 6: EGFr staining distant from the lesion in the margo plicatus after 48 hours of feed deprivation (Group 2). 20X magnification; bar in right corner=50 microns.
DISCUSSION

The distribution of epidermal growth factor receptor (EGFr) in equine gastric stratified squamous epithelia is similar to that previously described in human oral mucosa, esophagus, and skin. The greatest EGFr area and intensity are in the basal layer, which is where cell proliferation occurs and where EGFr messenger RNA has been identified. Receptor area and intensity diminish as cells lose their growth potential and become more differentiated toward the luminal epithelial layers. The distribution and number of epidermal growth factor receptors in skin is related to epithelial cell growth, which indicates that the EGF receptor and an EGF-like molecule are important in regulating cellular proliferation. Several growth factors utilize the EGF receptor, including EGF, TGF-alpha, and heparin-binding EGF.

The results of the study reported here imply that luminal epidermal growth factor does not have direct contact with the receptor in intact squamous mucosa, although cells exhibiting EGFr staining in the damaged erosion bed are exposed to luminal contents. Because the greatest receptor area and intensity were in basal cells and in cells adjacent to capillaries, the results suggest that the origin of the EGFr ligand is not the gastric lumen, but rather capillaries in the
lamina propria extending into the epithelium. The ligand may be transported in blood, or could be produced locally in capillary endothelial cells. EGF has been identified by immunohistochemical staining in endothelial cells of capillaries in normal esophageal squamous mucosa.\textsuperscript{159}

There were more capillaries apparent in the two groups of horses with gastric lesions, and they appeared to penetrate layers of the epithelium closer to the lumen as cellular proliferation occurred. Epithelial capillaries at 96 hours of feed deprivation had significantly greater EGFr area in adjacent cells than the normal or 48 hour group. Capillaries extending into the epithelium from the lamina propria appeared to form a "scaffold" upon which epithelial projections formed (Figure 6). Angiogenesis and adequate blood supply are crucial for wound healing, and these capillaries appeared to provide a framework for mucosal proliferation to take place.

We did not address the location of epidermal growth factor in equine gastric stratified squamous mucosa. It is possible that the growth factor gains access to the receptor by a paracrine mechanism. In normal human oral squamous mucosa, EGF has been found in connective tissue subjacent to the epithelium and it has been suggested that a paracrine mode of ligand-receptor interaction exists.\textsuperscript{116} It is also possible that TGF-alpha is
the principal ligand that binds to the EGF receptor in the squamous tissue of the alimentary tract. TGF-alpha in humans is expressed in the cytoplasm of stratified squamous epithelial cells of the esophagus. 

In the study reported here, there were significant differences in EGFr area and intensity associated with equine gastric squamous lesions when compared to non-ulcerated mucosa. There was greater area occupied by the receptor in the ulcer/erosion margin primarily at 96 hours feed deprivation. Increases in EGFr area were most pronounced in the squamous epithelium from the lesser curvature of the stomach, where ulceration tended to be more severe, which was probably due to greater exposure to peptic secretions because of the anatomic configuration of the equine stomach. By 96 hours of feed deprivation, the increase in EGFr area found in the ulcer/erosion margin was significantly greater than at 48 hours, which was coincident with the appearance of increased margin thickness seen endoscopically and microscopically between examinations after 48 and 96 hours of feed deprivation.

The greater EGFr area was not accompanied by darker EGFr intensity at any stage of ulceration. To the contrary, EGFr intensity at both stages of peptic damage was significantly less than normal mucosa (erosion bed of Group 2 and 3 margo
plicatus right, and ulcer/erosion margin, erosion bed, and distant from Group 2 and 3 lesser curvature). This could be due to an increase in the number of cells expressing the receptor in a given sample (greater EGFr area), but not greater receptor expression per cell. Since EGFr intensity is related to the three-dimensional receptor density within the tissue samples, a greater number of cells in a given area may alter the two-dimensional appearance of intensity. Staining differences between slides may also have influenced intensity characteristics.

There are differences in basal and stimulated EGFr density between squamous and glandular mucosa of the alimentary tract. Squamous epithelia of the upper gastrointestinal tract is highly proliferative, and EGFr is expressed prominently in those tissues. In this report, %EGFr area in the normal equine squamous fundus of the stomach was 67% (basal layer), decreasing to 27% closer to the gastric lumen. Similarly, %EGFR area in the normal human esophagus was 29.5%. 158

In contrast, gastric glandular mucosa is highly differentiated, therefore basal expression of EGFr in normal tissue is minimal. Glandular mucosa from sections taken at the margo plicatus from horses in this report showed little or no staining for EGFr. Likewise, %EGFr area in glandular mucosa of
Laboratory animals was only 0.51%\textsuperscript{12}. But when stimulated to proliferate under adverse peptic conditions, glandular mucosa can demonstrate dramatic changes in EGFr density to equal that of the constantly proliferating squamous epithelial mucosa. Glandular mucosa of rodents showed a 75-fold increase in %EGFr area (0.51% to 38.8%) in response to gastric ulceration,\textsuperscript{12} whereas inflamed human squamous esophageal mucosa had a 1.5 fold increase in %EGFr area (29.5% to 43.1%).\textsuperscript{198}

Interestingly, this study found significantly less EGFr area and intensity in the erosion bed of horses with gastric lesions after 48 hours of feed deprivation compared to normal gastric epithelium. The decrease in EGFr area was not evident in Group 3 horses (96 hours of feed deprivation). This decrease early in the course of ulceration could be due to receptor depletion, or less likely, receptor down-regulation. The erosion bed was exposed to excessive hydrochloric acid, which can lead to progressive damage to cells containing the receptor.

The basal and parabasal layers of the erosion bed also had direct contact with gastric luminal contents, and there could conceivably have been additional binding of luminal/salivary EGF to the receptor in the erosion bed that led to more rapid internalization and depletion of the receptor than would have
occurred in the margin or distant from the lesions. Erosion bed EGFr area was less in relation to the margin and distant from the lesion, also supporting receptor depletion. By 96 hours, EGFr area in the erosion bed (lesser curvature) was actually increased compared to normal tissue and the 48 hour group, implying that more time was required for increased receptor expression in the erosion bed than the margin or distant from the lesions due to temporary receptor depletion. Receptor down-regulation typically occurs in the later stages of wound repair and may serve as a mechanism to limit growth factor-mediated proliferation,\(^{182}\) therefore it would be less likely at early stages of gastric ulceration.

The greater receptor area in the erosion bed at 96 hours may reflect proliferation and migration of new cells to the middle epithelial layers damaged by peptic secretions. The lag period in response suggests that early initiation of cellular proliferation at the margin is important in supplying cells for re-epithelialization of the erosion bed.

This study also illustrated that greater EGFr area compared to normal tissue can occur a considerable distance away from the lesion (>1 cm). This indicates the extent of the epithelial proliferative response as reparative processes are initiated. Similar to the ulcer/erosion margin, increases in receptor
area distant from the lesion occurred very early in the process of peptic injury, emphasizing the rapidity with which healing processes begin. This was also supported by the significantly greater number of mitotic figures present in all areas of gastric lesions as early as 48 hours of feed deprivation compared to normal mucosa. Mitotic activity was most pronounced in the ulcer/erosion margin, which is where the greatest epithelial thickening occurred, but increased mitotic figures were also present as far away as 14 millimeters from the lesion.

CONCLUSIONS

Similar to other wounded squamous epithelia, ulcerated equine gastric stratified squamous mucosa exhibits significant changes in epidermal growth factor receptor expression. The greater receptor area strongly suggests that EGF and/or TGF-alpha play an important role in gastric ulcer and erosion repair in the horse. This is supported by the greatest presence of receptor on cells of the epithelial proliferative zone, which indicates that these cells are targets for EGF/TGF-alpha.

The results of this study also parallel findings in experimental ulceration of gastric glandular mucosa of
laboratory animals, in which the greatest increases in EGFr density occurred at the ulcer margin and up to 1 cm from the lesion.\textsuperscript{12,154} Because this study had a total protocol duration of 8 days, it was not possible to determine the duration of increased EGFr expression in gastric squamous mucosa of the horse, although increases observed in ulcerated rodent glandular mucosa were present even after ulcers were healed (up to 21-25 days).\textsuperscript{12,154}

Lastly, this study suggests that the stimulation of processes that promote healing of gastric ulcers in the horse occur almost at the same time as lesions are formed. Ulcers began healing while still subjected to peptic conditions that encouraged their formation. There are numerous contributors to wound healing, and growth factors are important components of this dynamic process. Hopefully, it will be possible to transform our continually progressing knowledge about growth factor effects into clinically useful, pharmacologic advancements in ulcer treatment and prevention.
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