Epidermal growth factor receptors in the oesophagus

J Jankowski, S Murphy, G Coghill, A Grant, K G Wormsley, D S A Sanders, M Kerr, D Hopwood

Abstract

The quantity and distribution of epidermal growth factor receptors (EGF-R) in oesophageal mucosa was studied in the oesophagus in order to determine its role in oesophageal disease. Fifty five biopsies were taken from different levels of the oesophagus in 25 consecutive patients undergoing endoscopy. Another group of eight patients with histologically proven Barrett's oesophagitis had a biopsy taken from the area of columnar lined oesophagus. A peripheral, membranous pattern was seen predominantly confined to the basal and immediately suprabasal cells in all of the first group of patients. In the superficial cells a few granular cytoplasmic structures were positive. All patients with Barrett's oesophagitis showed EGF-R staining of the surface epithelium. A computerised planimeter was used to determine the proportion of stained areas of squamous cells which were expressed as a percentage of the total area of squamous cells. The difference in the area of cells stained for EGF-R between normal and inflamed oesophageal mucosa (29.5% and 43.1% respectively) was significant ($p < 0.001$).

Epidermal growth factor is a mitogenic polypeptide which plays an important role in wound healing, maintenance of tissue integrity, and maturation.^{1,2} This ligand binds to epidermal growth factor receptors, which are integral plasma membrane glycoproteins, possessing intrinsic tyrosine specific protein kinase activity.³⁻⁶ This receptor has been shown to be involved in cellular proliferation and differentiation as well as participation in oncogenesis⁷⁻¹⁹ when the former processes become abnormal.

The epidermal growth factor receptor is expressed throughout the human intestine including the oesophagus⁹ but its presence in oesophageal mucosa has not been studied in detail. The present study therefore examines the presence of epidermal growth factor receptors in normal oesophageal mucosa and in the mucosa of patients with oesophageal reflux disease. We also assessed the clinical usefulness of epidermal growth factor receptors in confirming the diagnosis of oesophageal reflux disease.

Methods

PATIENTS

We studied 33 consecutive patients attending an oesophagoscopy clinic. Twenty five patients had normal or inflamed squamous oesophageal mucosa: 14 were women and 11 men, with a

mean age of 55 years (range 23-77). Eight additional patients had Barrett's mucosa, five women and three men (median age 63 years (range 43-79)).

Biopsies were taken from different levels of the oesophagus (20 cm and 30 cm from the incisors and at the gastrooesophageal junction (36-42 cm)). Eleven patients had biopsies taken from all three levels and another 30 had random biopsies from one level only (including eight patients with Barrett's mucosa). One of us (DH) graded all sections according to the severity of the histological changes (Jarvis criteria).²⁰

Immediately adjacent to the sample taken for histological examination, a second sample was taken and immunohistochemically processed to show epidermal growth factor receptors. The biopsy was immediately snap frozen in the endoscopy room. Six micrometre cryostat sections were cut. Epidermal growth factor receptor detection involved the avidin biotin peroxidase complex method.²¹ The epidermal growth factor receptor 'monoclonal antibody - type 1' was supplied by Oncogene Science (Manhasset, New York, USA). The monoclonal antibody to epidermal growth factor receptors is an IgG mouse variety and was diluted to 1:50.

Negative control sections of oesophageal mucosa, exposed to avidin biotin alone and Mab epidermal growth factor receptors alone showed no detectable staining pattern. Oesophageal squamous carcinoma was used as positive control tissue as this carcinoma expresses more epidermal growth factor receptors than any other tissue.

A computerised planimeter (Sonographics, USA) and Imagan-2 software (Kompira, UK) was used to determine the proportion of stained areas of squamous mucosa as a percentage of the total area of squamous mucosa by two independent observers (JJ and SM).

The biopsies from Barrett's columnar lined oesophagus were graded for dysplasia (Riddell criteria).^{22,23} The epidermal growth factor receptor content of the Barrett's mucosa was assessed by the method described by Koretz.²⁴

We utilised the techniques of flow cytometry in an attempt to correlate the density of cell membrane epidermal growth factor receptor expression with cell size. Five patients each had six biopsies removed from the squamous oesophagus and incubated overnight in 1% trypsin in phosphate buffered saline at 4°C. The cells were then agitated for one minute by repeated suction into a narrow bore pipette. Cells were labelled with epidermal growth factor receptor antibody at 1/100 dilution for 30 minutes at room temperature. They were washed in phosphate buffered solution and labelled with fluorinated

University Departments
of Clinical
Pharmacology, and
Pathology, Ninewells
Hospital, Dundee
J Jankowski
S Murphy
G Coghill
A Grant
K G Wormsley
D S A Sanders
M Kerr
D Hopwood

Correspondence to:
Dr J Jankowski,
Histopathology Unit, Imperial
Cancer Research Fund,
35-43 Lincolns Inn Fields,
London WC2 3PN

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Figure 1: (a) Normal oesophageal tissue showing epidermal growth factor receptor immunohistochemical staining. (b) Normal oesophageal tissue showing intracellular epidermal growth factor receptor particulate staining. (c) Positive epidermal growth factor receptor immunohistochemistry in a section with Barrett's mucosa.

the groups and within the groups. All values were assessed to 95% confidence intervals.

Results

HISTOLOGICAL APPEARANCES

Thirty two sections were reported as normal and 23 as showing inflammation of the squamous mucosa. Eight patients had well differentiated columnar lined oesophageal mucosa. Six being of the cardiac type and two of the intestinal type.²³

DISTRIBUTION OF EPIDERMAL GROWTH FACTOR RECEPTOR

Figure 1a shows the pattern of distribution of epidermal growth factor receptors in oesophageal squamous cell membranes. Staining is strongly positive in the basal cell compartment and decreases in amount through the prickle cell layer and in the prolongations around the papillae, being absent from the cell membranes in the upper functional or superficial cells (Fig 1a). This membranous' staining pattern was completely circumferential involving the basolateral and apical cell surfaces. There is focal particulate epidermal growth factor receptor positivity in some of the prickle and superficial cells, possibly in lysosomes or other intracellular bodies. At higher magnification some small epidermal growth factor receptor positive bodies were noted in the cytoplasm of the basal and prickle cell compartments. These may represent organelles involved in the synthesis and packag-

mouse secondary antibody at 1/500 dilution for 20 minutes at room temperature. Cells were fixed in 2% paraformaldehyde. A Becton-Dickinson FACSCAN flow cytometer was used to assess the relative epidermal growth factor receptor fluorescence as function of cell volume. Ten thousand labelled cells were counted from each patient. The 'consort-30' program was used to assess fluorescence and the 'LYSYS' program used to calculate the statistics (both Beckton-Dickinson software).

STATISTICAL ANALYSIS

Analysis of variance was used to assess the significance of the distribution of values between

Figure 2: Epidermal growth factor receptor immunohistochemistry at different levels of normal oesophagus.

ing of the receptor (Fig 1b). Only occasional cells in the lamina propria and its papillary extensions were positive for epidermal growth factor receptor.

EPIDERMAL GROWTH FACTOR RECEPTOR STAINING AT DIFFERENT LEVELS OF THE OESOPHAGUS
In normal mucosa there were 11 biopsies from 20 cm; 11 from 30 cm; and 11 from the gastrooesophageal junction. Figure 2 graphically illustrates that the mean epidermal growth factor receptor counts and the 95% confidence intervals at different oesophageal levels overlap in normal individuals.

EPIDERMAL GROWTH FACTOR RECEPTOR STAINING IN OESOPHAGITIS
The proportion of positively stained oesophageal epithelium was significantly greater in inflamed mucosa compared with normal uninflamed mucosa, staining positively 43% of the total area of inflamed squamous mucosa compared with a normal value of 29% (Fig 3).
The separation between normal oesophageal mucosa and inflamed oesophageal mucosa by planimetry was highly significant ($p<0.001$).
There was positive staining of superficial epithelial cells in glandular areas of all sections of the columnar lined mucosa (Fig 1c). The staining is mainly membranous in nature and present on apical and basolateral surfaces although there is also intracytoplasmic speckling.

FLOW CYTOMETRY RESULTS
In all five patients analysis by flow cytometry showed that there was no statistical difference between the fluorescence of the larger squames compared with the smaller squames ($p=0.4$) (Fig 4). This finding indicates that both the larger superficial cells have a similar number of

epidermal growth factor receptors expressed on the cell membrane as the smaller basal cells.

Discussion
The epidermal growth factor receptor is a glycoprotein which has ligand dependent tyrosine kinase activity.⁴ The N-terminal has N-linked oligosaccharide chains which may confer species specific differences in ligand binding.²⁵⁻²⁹ Epidermal growth receptor type-1 monoclonal antibody has been shown to bind to the 14 amino acids from Ala-351 to Asp-364 of the mature human epidermal growth factor receptor.³⁰ In addition, there are 16 other monoclonal antibodies which bind to different epitopes on the epidermal growth factor receptor.³⁰
The oesophageal squames normally have 20 000 to 200 000 epidermal growth factor receptors per cell²⁹ and when squamous carcinoma develops this is associated with even greater expression of epidermal growth factor receptors.¹¹ It has been suggested previously that all receptors can be demonstrated by immunohistochemistry (both high and low affinity receptors).¹⁵
The present immunohistochemical study shows that normal oesophageal mucosa has epidermal growth factor receptors in the basal and prickle cell layers, on the cell membranes, giving a 'chicken-wire' appearance in sections. This pattern of staining has been described previously by Ozawa *et al.*¹¹ The more mature superficial epithelial cells are minimally stained immunohistochemically although there are a few immunostaining particulate bodies in the cytoplasm of these cells which probably represent lysosomes containing epidermal growth factor bound to its receptor.²⁴ This pattern of expression of epidermal growth factor receptors is uniform, irrespective of site in the oesophagus.
Flow cytometry has indicated that the superficial epithelial cells have just as many epidermal

Figure 3: Epidermal growth factor receptor immunohistochemistry in normal and inflamed oesophageal tissue.

Figure 4: Scattergram of epidermal growth factor receptor expression on 10 000 oesophageal squamous cells. x-axis fluorescence per cell, y-axis cell size.

growth factor receptors per cell as smaller cells. The discrepancy between the results of flow cytometric measurements and histological staining for epidermal growth factor receptors expression in the superficial layers merely reflects the greater sensitivity of laser aided fluoroscopy (flow cytometry) compared with light microscopical methods of immunohistochemistry.³¹ It has recently been reported that the amount of binding of radiolabelled human epidermal growth factor is similar in basal and superficial cells in the oesophagus.³² The higher density of receptors on the surface of the basal cells, however,^{11 32 33} not only explains the immunohistochemical findings but also possibly explains, in part, the greater mitotic activity of basal cells. Dimerisation of receptors is important in determining the magnitude of the subsequent intracellular actions of epidermal growth factor after binding of the ligand. Cells without a dense distribution of membrane receptors may have less epidermal growth factor induced dimerisation and therefore experience less of the subsequent intracellular actions of the peptide.³⁴

We have recently shown that epidermal growth factor may also be found in the capillary endothelium of the submucosa, immediately adjacent to the cells with dense epidermal growth factor receptor expression.³⁵ It seems possible, therefore, that the normal oesophageal mucosal basal layer has greater proliferative activity because it has maximal epidermal growth factor receptor density and a rich nearby source of epidermal growth factor. Our study indicates that inflamed oesophageal mucosa has significantly greater density of epidermal growth factor receptor expression than normal mucosa, a finding perhaps related to the fact that cellular proliferation is increased in oesophagitis.³⁶ Positive epidermal growth factor receptor staining is also present in many epithelial cells from the well differentiated columnar lined (Barrett's) oesophagus, a finding which may be significant because epidermal growth factor receptor expression may be associated with cancer and

oncogenesis. In this connection, we have recently reported the increased expression of epidermal growth factor receptors in intestinal type Barrett's mucosa and adenocarcinoma arising in Barrett's mucosa.³⁷ In addition, production of abnormally great amounts of epidermal growth factor receptors has been shown in many squamous cell carcinomas, including those of the oesophagus,¹¹ and buccal cavity.^{12 13} In cancers of tissues such as breast and bladder, increased epidermal growth factor receptor levels have significant prognostic correlation with the pathological characteristics, such as tumour invasion.¹⁵⁻¹⁷ Whether this is also true for oesophageal carcinomas is not yet clear.¹¹ It has been reported, however, that the squamous cells of oesophageal cancer expresses more epidermal growth factor receptor than any other tumour in vivo.¹⁸

The evidence for epidermal growth factor receptor related oncogenesis in various tumours is presented in reports showing that a ligand independent mutant of epidermal growth factor receptor derived from the oncogene c-erbB has been associated with the formation of transformed epithelial cells^{39 40} and also that many squamous cell carcinomas^{11 12 14 41} and adenocarcinomas, including a case of Barrett's carcinoma overexpress the epidermal growth factor receptor gene.^{3 24 42-44} In addition, some carcinomas have a poorer prognosis and/or a greater chance of recurrence if they are epidermal growth factor receptor positive.^{15 16 45}

It seems possible that epidermal growth factor receptors represent a histological marker of the increased cell proliferation in the oesophagus, which results from gastrooesophageal reflux.^{46 47} The stimulus to increased cell proliferation provided by refluxed gastric contents may act through epidermal growth factor receptor gene amplification or increased epidermal growth factor receptor gene transcription,^{6 48} while in Barrett's mucosa, epidermal growth factor receptor expression may represent activation of a proto-oncogene, so that subsequent gene mutations or deletions could result in neoplastic growth.⁴⁹ It is also possible that coexpression of epidermal growth factor receptor with the ligands transforming growth factor-alpha and/or epidermal growth factor stimulate other oncogenes such as c-fos and c-myc as has been shown in other gastrointestinal tumours.⁵⁰⁻⁵²

This present study has shown that epidermal growth factor receptor staining may be a useful adjunct to histological examination in the diagnosis of oesophagitis. Further studies are underway to assess the prognostic value in Barrett's oesophagus.

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