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The expression of Lewis^x on carcinoembryonic antigen (CEA)-related glycoproteins of normal and inflamed oesophageal squamous mucosa

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Carcinoembryonic antigen (CEA)-related antigens were detected histologically in normal and inflamed oesophageal squamous mucosa using polyclonal anti-CEA antisera and monoclonal antibodies recognizing CEA or NCAs (non-specific cross-reacting antigens). Expression was limited to the surface of more mature squames. Immunoblotting of detergent extracts of oesophageal mucosa separated on polyacrylamide gels using polyclonal anti-CEA antisera showed a number of CEA-related proteins, of 195, 145, and 80 kDa. CEA-specific monoclonal antibodies recognized only the 195-kDa glycoprotein. The lower molecular weight species were recognized by anti-NCA antibody DD9 and a CD66 antibody. The carboyhydrate antigen, Lewis^x (Le^x, CD15), previously shown to be a marker of mature squames, was present predominantly on a subpopulation of the 195-kDa antigen and was demonstrable on the higher molecular weight component of a doublet recognized by the CEA antibodies. Expression of Le^x carbohydrate antigens in inflamed oesophageal squamous mucosa was shown to be significantly reduced relative to the expression seen in normal tissue. A suprabasal layer of CEA-positive, Le^x-negative cells became apparent in inflamed tissue showing altered glycosylation of the CEA under these conditions. It is postulated that CEA plays a role in maintaining the integrity of the squamous mucosa.

Keywords: carcinoembryonic antigen, CD15, Lewis^x, oesophagus, squamous mucosa

Introduction

The oesophageal squamous epithelium plays an important role in prevention of mechanical and chemical damage. The epithelial surface must resist the attack of acid and digestive enzymes from the stomach and duodenum. Oesophagitis and reflux are common conditions affecting 40–50% of the population [1], with increased occurrence associated with overindulgence at certain times of the year. More serious forms of the disease which occur can be treated with H_2 blockers. Complications can extend to Barrett's oesophagus and oesophageal carcinoma [2], which have increased in incidence over the past few years [3]. Relatively little attention has been paid to mechanisms which contribute to the barrier function of the mucosa and the response to regurgitating gastric and duodenal contents.

We have previously demonstrated that different morphological patterns of damage occur with gastric and duodenal juices [4, 5]. A common response, however, was noted in the decrease in number of functional cells and an increase in the basal and prickle cells. The increase in immature cells can be seen histologically as an increase in the basal/prickle cell layers and extension towards the oesophageal lumen of the capillaries [6]. At the same time there is a decrease in the amount of glycogen found in the mature cells.

In the stratified squamous epithelium of normal oesophagus or cervix, carbohydrate structures appear to be added to membrane glycoprotein chains in stepwise fashion as the basal cells mature. The

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fucosyl residues which define the Lewis blood group antigens are some of the most peripheral of these structures [7]. In contrast to the cervix, there is little sialylation of the carbohydrate chains in the oesophagus [7]. During inflammation, the expression of the related carbohydrate structures, Lewis^x (Le^x, CD15) and Lewis^a (Le^a), found only on mature cells, decreases with the decrease in the proportion of mature cells [6].

CEA is a heavily glycosylated protein which has been used widely as a serum marker of neoplasia. In tumours, CEA is frequently associated with a number of carbohydrate 'tumour markers', including those related to the Lewis blood group antigens. Recent work has shown that CEA is one member of a large family of proteins found in normal as well as neoplastic tissues. We have shown that, in the cervix, Le^x and sialylated Le^x are coexpressed on CEA-related glycoprotein [8]. We have also recently demonstrated the presence of CEA-related glycoproteins in the oesophagus [9]. The oesophagus represents a more accessible tissue to study the differentiation of wet squamous epithelium. We have therefore investigated the expression of CEA-related molecules on normal and inflamed oesophagus. We have found a surprising complexity in the expression of CEA-related molecules. A subpopulation of the higher molecular weight CEA expressed all of the Le^x. The known role of CEA family members as adhesion molecules suggests that they may play a role in the formation of the oesophageal permeability barrier, supplementing the desmosomes which have been shown to hold the squames together.

Materials and methods

Antibodies used in this study were:

(a) Polyclonal rabbit anti-CEA (Dako, High Wycombe, UK) used at 1:500 dilution.
(b) Monoclonal anti-CEA 'A5B7' (Dako), which does not recognize NCAs when used at 1:300 dilution [10].
(c) Monoclonal anti-CEA '198' (a gift from Dr M. R. Price, CRC Laboratories, University of Nottingham, UK) tissue culture supernatant used neat [11].
(d) Monoclonal anti-CEA IM10 6.2 and IM10 13.1 (Scottish Antibody Production Unit, Carluke, UK) ascites used at 1:40 and 1:80 dilution respectively.
(e) Monoclonal anti-NCA 50/90 'DD9' (a gift from Dr A. Grant, Department of Clinical Biochemistry, St. Georges Hospital and Medical School, London, UK) used as neat tissue culture supernatant [12].
(f) CD66-classified rat monoclonal antibody YTH-71.3 (a gift from H. Waldmann, Department of Pathology, Cambridge University, UK) raised against leucocytes, used at 1:100 dilution [13].
(g) Anti-Lewis^x, CD15-classified mouse IgM monoclonal antibody MC2, raised in this laboratory, used at 1:250 [14].

For controls in immunohistochemistry or immunoblotting, normal rabbit serum, normal rabbit IgG, tissue culture supernatant or ascitic fluid containing an irrelevant monoclonal antibody were used at the same dilution as the specific primary antibody.

Immunohistochemistry

Staining of 5-μm sections of routinely paraffin-processed oesophageal biopsies was carried out using antibodies against CEA or related antigens. Binding of rabbit antibodies was detected using goat anti-rabbit second antibody with an avidin–biotin complex (ABC) as described by the manufacturers (Vectastain Kit, Vector Laboratories, Peterborough, UK). Binding of mouse or rat monoclonal antibodies was detected using rabbit anti-mouse second antibody with a streptavidin ABC (Dako) as described by the manufacturers.

Immunoblotting

Endoscopically and histologically normal or inflamed oesophageal mucosa was used, obtained from organ donors or pinch biopsies. The contamination rate of mesenchyme in these biopsies is about 5% [15]. The specimen was chopped finely, resuspended in phosphate-buffered saline (PBS) at a concentration of 100 mg wet tissue/ml and then homogenized thoroughly using a Polytron homogenizer at half-speed for 2 min. Triton X-100 was added to a final concentration of 1% (v/v) and the extract rotated for 1 h at 4°C. After centrifugation at 13 000 *g* for 3 min (MSE microCentaur) the supernatant was removed and used for further study.

Proteins from the oesophageal mucosal extracts were separated by SDS–polyacrylamide gel electrophoresis on 10% polyacrylamide slab gels by the method of Laemmli [16]. For immunoblotting, approximately 100 μg of protein was run per lane, except when subsequent immunoblotting was

to be carried out with multiple antibodies, when 1.5 ml of extract (3 mg of protein) was loaded onto gel having a single, large (15 cm), sample well. After electrophoresis, the gel proteins were transferred to nitrocellulose by the method of Towbin *et al.* [17] except that the transfer buffer contained only 15% methanol. The dried nitrocellulose was cut into narrow strips and the remaining protein binding sites of the nitrocellulose were then blocked by incubation with 5% skimmed milk (Marvel) solution in PBS. The blots were incubated with antibodies from the list above (at the given dilutions in skimmed milk/PBS) for 2 h, then washed for 3 × 10 min in PBS before incubation with the secondary antibody (alkaline phosphatase-conjugated anti-mouse IgG, anti-mouse IgM or anti-rabbit IgG from Sigma) at 1:500 dilution in milk/PBS. After washing three times in PBS the blots were then stained using substrate containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 3.3 μg/ml) and nitro-blue tetrazolium salt (NBT; 6.6 μg/ml) in 0.1 M Tris–HCl, pH 9.5/0.1 M NaCl/5 mM $MgCl_2$.

Immunoprecipitation

Purified IgG from the anti-CEA antiserum was dialysed into PBS, then conjugated to cyanogen bromide (CNBr)-activated Sepharose at 0.5 mg of IgG per ml of resin according to the manufacturer's instructions. A 200 μl volume of the Triton X-100 extract of oesophageal biopsies was incubated with an equal volume of the antibody–Sepharose for 2 h at 4°C and then the resin packed into a small column. The resin was washed with 10 volumes of PBS/0.1% Triton X-100, followed by five column volumes of 0.75 M NaCl/0.1% Triton X-100/20 mM sodium phosphate pH 7.4 and finally 10 column volumes of PBS/0.1% Triton X-100. The bound antigen was then eluted with 0.2 M acetic acid/0.1% Triton X-100 and the eluted proteins were neutralized with 35 μl of 3 M Tris–HCl, pH 8.7/0.1% Triton X-100 per 200 μl of acetic acid.

Results

Immunohistochemistry

All anti-CEA and anti-NCA antibodies gave similar patterns of staining in immunohistochemical sections of normal squamous mucosa (10/10). Serial samples showed each antigen to be expressed to a similar depth in the epithelium, giving a strong membranous 'chicken-wire' pattern on a broad zone extending from the prickle cells right to the surface. The more mature cells showed only membrane staining. At higher power this gave a characteristic 'tramline' appearance in which each cell was stained but spaces between the cells were not. These included the most superficial cells and the underlying functional cells. Representative sections from a single donor are shown in Figure 1A. Some specific intracellular granular staining was observed in cells of the functional layer. Some antibodies gave weak cytoplasmic staining. In each case this was non-specific staining since it was not

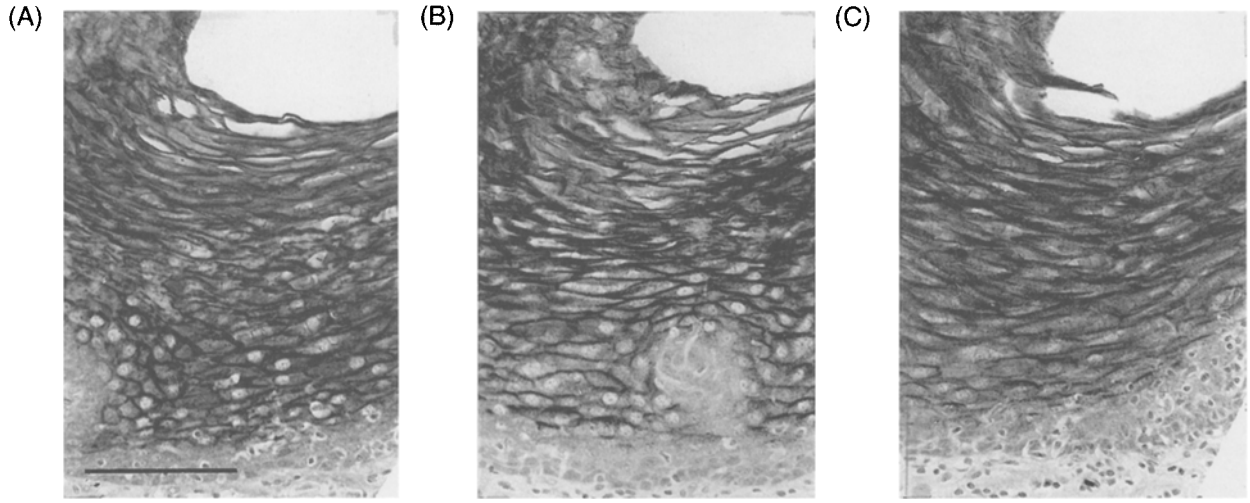

Figure 1. Expression of CEA in normal oesophageal mucosa. Sections of oesophageal mucosa 5 μm thick obtained from an organ donor were stained using anti-CEA antibodies. (A) Polyclonal rabbit anti-CEA; (B) monoclonal A5B7; (C) monoclonal IM10 13.1. With all antibodies, staining extends through the suprabasal layer (magnification × 150). A = 46 × 66 mm, B = 45 × 67 mm, C = 46 × 65 mm. Bar = 150 μm.

removed when specific antibody was removed from the preparation by absorption on CEA–Sepharose. The surface staining was never observed with control antibodies. As we have previously shown, the same pattern of expression in the normal oesophageal mucosa was seen for the Le^x and Le^a epitopes [6].

In inflamed tissue the basal compartment was extended. Numbers of papillae extending up into the epithelium and the proximal basal cells were CEA negative (10/10). In contrast to normal tissue, where CEA and Le^x were coexpressed, it was evident that CEA expression extended further into the basal layer (Figure 2A) than the more superficial staining seen with anti-Le^x antibody (Figure 2B). Similar results were obtained for biopsies from 10 patients with suspected oesophagitis. The staining by anti-CEA antibodies was always very similar. However, the depth of the superficial layer which expressed Le^x varied in thickness. In the most extreme cases only the very superficial cells expressed Le^x. The section shown in Figure 2B represents a intermediate case.

Immunoblotting of CEA-related glycoproteins in oesophageal tissue extracts

Detergent extracts of membrane proteins from normal oesophagus were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% gels under reducing conditions. Several anti-CEA and anti-NCA antibodies were used in immunoblotting as probes to identify CEA and related glycoproteins. Polyclonal anti-CEA immunoglobulins gave a strong reactivity with a glycoprotein giving a broad complex band in the region of 180–200 kDa, as well as a further three bands in the region of 120–160 kDa. The polyclonal anti-CEA immunoglobulin also recognized a protein of around 80 kDa (Figure 3, lane 8). Several anti-CEA monoclonal antibodies recog-

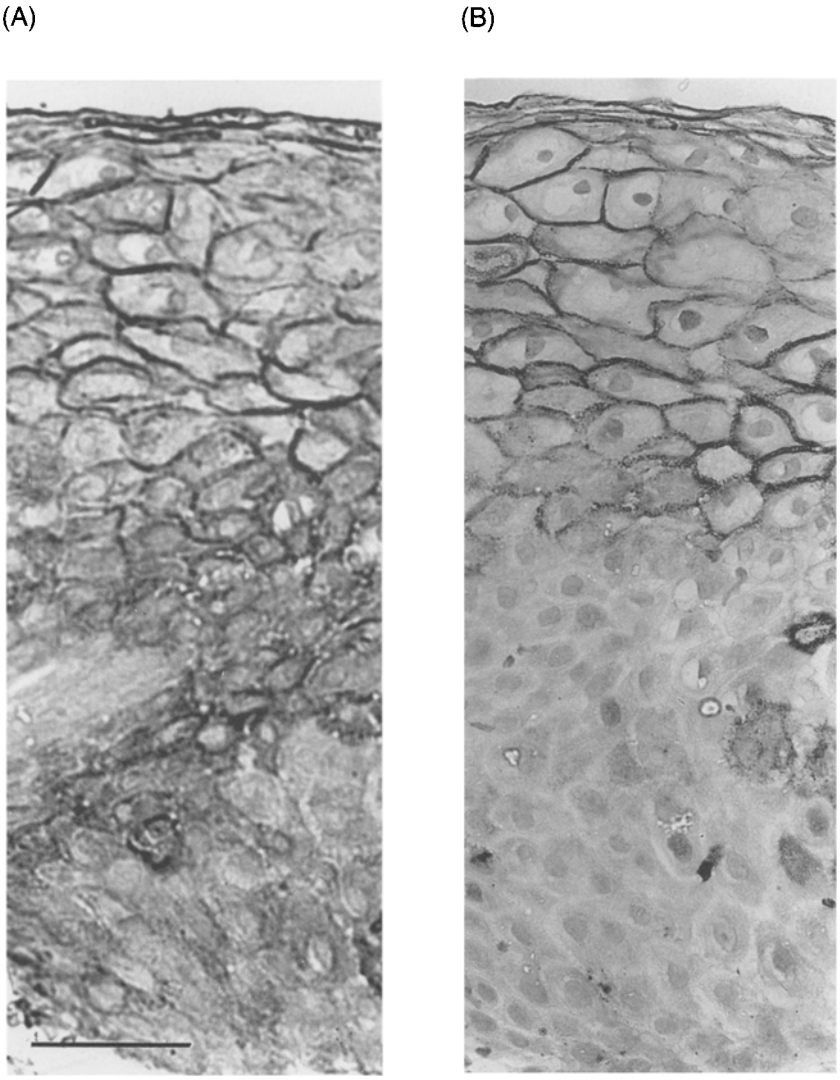

Figure 2. Expression of CEA and LE^x in inflamed oesophageal mucosa. Serial 5-μm sections from a pinch biopsy of an inflamed oesophagus (A) stained with polyclonal rabbit anti-CEA and (B) stained using anti-Le^x. The staining for Le^x is clearly more superficial than that for CEA (magnification × 200). A = 56 × 127 mm, B = 56 × 129 mm. Bar = 300 μm.

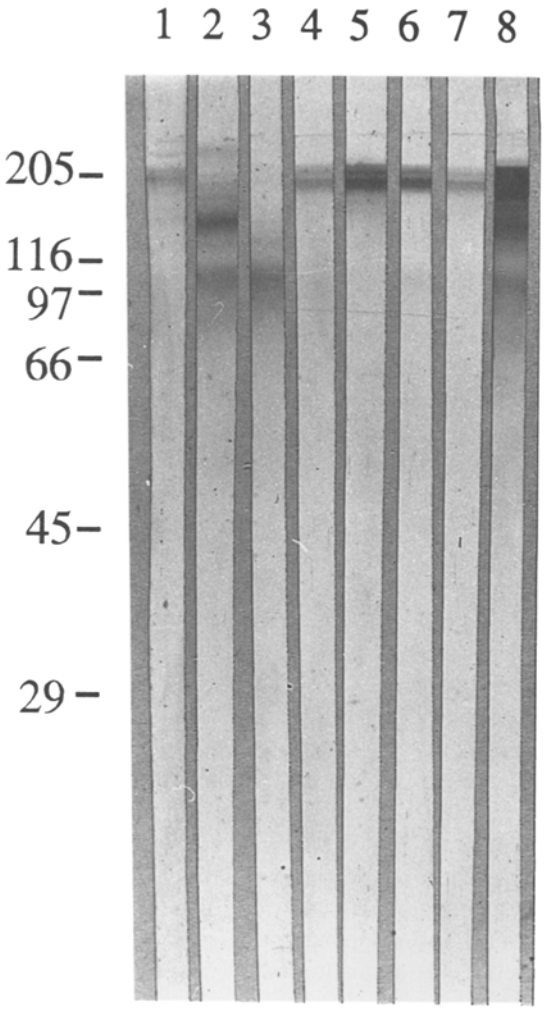

Figure 3. Immunoblot of detergent extract of normal oesophageal mucosa. Oesophageal extract glycoproteins were resolved under reducing conditions on 10% SDS–PAGE and blotted with the following antibodies: 1, anti-Le^x; 2, YTH71.3; 3, DD9; 4, IM10 13.1; 5, IM10 6.2; 6, 198; 7, A5B7; 8, polyclonal rabbit anti-CEA. M_r markers shown on the left are, from top to bottom: myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 29 kDa.

nized a doublet at around 185–195 kDa, and although there were different intensities of staining the specificity of these antibodies appeared to be identical. The staining pattern was apparently more complex than that previously reported for cervical squamous epithelium [8] where only a 180–190 kDa band was detected by the polyclonal anti-CEA antiserum. The highest molecular weight CEA-related protein detected in cervix and oesophagus is of slightly higher molecular weight than standard colonic carinoma CEA (165 kDa in our hands). All of the CEA-related molecules give characteristically broad bands upon electrophoresis, possibly reflecting the range of different glycoforms of each species. The majority of protein bands detected by staining of the gels with Coomassie blue were as tight as would be expected for other glycoproteins. Any molecular weight determinations made for CEA-related molecules must therefore be regarded as approximations.

Monoclonal antibodies which recognize NCAs gave interesting but different patterns of reactivity. DD9, raised against pancreatic cancer, specifically recognized the 80-kDa glycoprotein also identified by the polyclonal anti-CEA (Figure 3, lane 3). YTH 71.3, a CD66 antibody which recognizes leucocyte NCA, recognized the same 80-kDa glycoprotein, and also, with greater intensity, a glycoprotein of 145 kDa, which was also recognized by the polyclonal anti-CEA (Figure 3, lane 2). Anti-Le^x monoclonal antibody MC2 recognized predominantly a single glycoprotein with the same mobility as the highest M_r band recognized by the anti-CEA antibodies, apparently corresponding to the larger of the two components recognized by the anti-CEA monoclonals (Figure 1, lane 1).

Immunoblotting of three extracts of biopsies of inflamed oesophagus gave very similar patterns of staining to normal tissue with all anti-CEA and NCA antibodies tested. For one specimen in which immunohistochemistry showed the presence of Le^x on only the most superficial cells, the immunoblots appeared to show the presence of less Le^x on the highest molecular weight CEA. Control antibodies gave no staining of immunoblots of normal or inflamed tissue.

Le^x is expressed by CEA-related molecules in the oesophagus

To confirm that Le^x was expressed predominantly on CEA, a normal oesophageal tissue extract was subjected to immunoprecipitation using anti-CEA antisera and the immunoprecipitated CEA-related proteins were probed by immunoblotting with anti-CEA immunoglobulin and anti-Le^x, MC2. Oesophageal CEA clearly expressed the Le^x determinant (Figure 4A, lanes 2 and 3). It was also apparent that only the highest M_r form of CEA carried the Le^x determinant.

Discussion

The CEA family is a large family of glycoproteins related structurally and antigenically to CEA, ex-

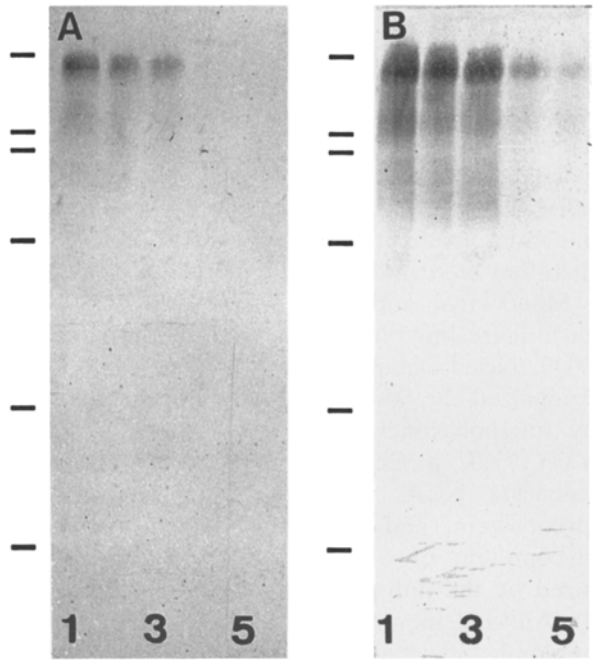

Figure 4. Immunoblot of anti-CEA-precipitated glycoproteins from normal oesophageal mucosa. Oesophageal CEA-related glycoproteins were precipitated using polyclonal rabbit anti-CEA immunoglobulins bound to Sepharose. The proteins were resolved under reducing conditions by SDS–PAGE using 10% polyacrylamide gels. Transferred proteins were immunoblotted with (A) anti-Le^x or (B) polyclonal rabbit anti-CEA. Samples were: 1, oesophageal extract; 2,3, proteins bound to and eluted from the resin; 4,5, non-absorbed proteins. Molecular weight markers as in Figure 3.

pressed in normal tissues as well as in carcinoma [18]. Polyclonal antisera to CEA react with many members of the CEA family, including the NCA-related antigens found on tumours and on granulocytes. The first NCA was discovered in fetal liver as a protein which cross-reacted with antibodies raised against CEA [19]. Since then, NCAs have been demonstrated on many tissues as well as in serum and amniotic fluid. NCA has also been shown to be present on polymorphonuclear cells and macrophages (but not monocytes) [20], although biochemical characterization of the myeloid NCAs has only recently begun [21–23]. The reason for the cross-reactivity between different members of the CEA family is the very high level of sequence homology between the different proteins, resulting in the same epitope being expressed on many different NCAs and CEA.

Our studies demonstrate the expression of glycoproteins recognized by anti-CEA antibodies on the surface and in intracellular granules of mature, normal suprabasal oesophageal squamous epithelial cells. Immunoblotting has demonstrated that a number of CEA-related molecules recognized by polyclonal anti-CEA antiserum are differentially recognized by monoclonal antibodies specific for different members of this family. The expression of the different CEA-related molecules was not altered greatly in inflamed tissue. Our studies further emphasize the wide expression of these molecules, showing clearly that, histochemically, CEA-treated molecules are in no way specific markers of malignancy.

Members of the CEA family are all heavily glycosylated. The core peptide of CEA itself is only 80 kDa, the mature glycoprotein being 180 kDa, carbohydrate therefore accounting for approximately 60% of the mass of the glycoprotein [24]. In colonic carcinoma CEA, the Le^x group, Galβ1 → 4(Fucα1 → 3)GlcNAcβ1 → R, accounts for a high proportion (35.1%) of the desialylated outer chain moieties [25].

The expression of the Le^x, CD15, epitope on the epithelial tissues which express CEA-related molecules is of interest, since both have been implicated in intercellular adhesion reactions. It is also interesting that CEA-related molecules carry this determinant in more than one tissue. The Le^x determinant has wide expression in normal non-haematopoietic tissues [26, 27], particularly gut epithelia and neoplastic tissues [5]. A decrease in expression is associated with development of cervical intraepithelial neoplasia, which reflects the changes in maturity of predominant cell types, and may be of use in characterization of certain tumours [28].

The present study shows that the Le^x epitope is expressed predominantly on only one glycoprotein in the oesophagus, the higher M_r glycoprotein of the doublet recognized by anti-CEA antibodies. It is therefore possible that at least one of the CEA-related molecules in the oesophagus has forms with different glycosylation patterns and that the glycosylation might be important in functional regulation. It is remarkable given the high degree of sequence homology between the CEA family members and their high level of glycosylation that only one member of the family expresses Le^x.

It has been shown that the Le^x antigen as well as Le^a and Le^b blood group antigens are present only on mature cervical or oesophageal squames [6, 7]. Here we show that differently glycosylated forms of CEA are associated with the stage of maturation of the squames. In the inflamed oesophageal mucosa, with an increased proportion of immature

cells, a reduced expression of the Le^x epitope on CEA has been demonstrated. It would appear that CEA lacking Le^x is produced by the suprabasal cells during inflammation of the oesophagus. However, the results of immunoblotting have to be interpreted with caution since the biopsy samples are small with a squamous cell layer only a few cells thick. It is therefore not possible to isolate cells from the different layers. In all but the most inflamed tissue, changes in expression of Le^x occur in only a narrow layer of suprabasal cells, thus immunoblotting will still detect material predominantly from the upper squamous layer, which is always Le^x positive.

It is likely that the similarities in carbohydrate expression of related proteins in different tissues are a reflection of the functional significance of the carbohydrate groups. Since these carbohydrate groups have been demonstrated to be involved in adhesion, and as CEA and related proteins also have a role in adhesion, it is possible that these molecules are also involved in the homotypic adhesion of mature squamous epithelia. The location of CEA in the glycocalyx of the cervical squamous epithelium [7] is consistent with a role as an adhesion molecule.

A role in adhesion for CEA-related molecules was first demonstrated by Oikawa *et al.* [29], who confirmed that CEA can be involved in homotypic adhesion. They demonstrated that transfectants expressing NCA will adhere to both NCA- and CEA-expressing cells. Recent studies have also implicated granulocyte NCA-160 in adhesion of granulocytes to endothelium [30, 31]. Le^x and sialylated Le^x undoubtedly play a key role in the adhesion of leucocytes to endothelium [32]. We have recently shown that NCA-160 is the major neutrophil glycoprotein expressing Le^x and sialylated Le^x [33]. Localization of CEA to the cell membrane of mature cervical squames is consistent with a role for these antigens in maintaining the integrity of the squamous mucosa, forming a sort of intercellular cement. CEA mediates Ca^{2+}-independent homotypic aggregation of cultured human colon carcinoma cells and can effect the homotypic sorting of cells in heterogeneous populations of aggregating cells [34]. Since several CEA family members [29, 34–37] as well as Le^x (reviewed in ref. 32) have been shown to be involved in adhesion interactions, it is possible that oesophageal CEA is involved in promoting the critical adhesion interactions which occur between the mucosal cells and that changes in the glycosylation of these proteins might be important in controlling the integrity of the mucosal barrier in health and inflammation.

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