SHORT COMMUNICATION

Immunohistochemical detection of epidermal growth factor receptors on human colonic carcinomas

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Epidermal growth factor (EGF) and transforming growth factor α (TGFα) are peptide growth hormones which are known to regulate the proliferation of a variety of cells (Carpenter & Cohen, 1979; Cohen, 1983; Marquardt et al., 1983) including the gastrointestinal cells in rodents (Al-Naffusi & Wright, 1982). Both of these peptides exert their influences via the EGF receptor, a 170 kDa phosphoglycoprotein located across the cell membrane (Carpenter & Cohen, 1979). The internal portion of this receptor bears a close relationship to the v-erb-B oncogene product (Downward et al., 1984), and it is likely that growth factors play a role in oncogenesis (Burgess, 1985).

Certainly, a number of human neoplastic cell types have been shown to express the EGF receptor to a variable degree (Gusterson et al., 1984), and there is evidence that increased expression is associated with a poor prognosis (Neal et al., 1985; Sainsbury et al., 1987). Little work has been done on colonic cancer, and although this tumour has been shown to carry the receptor (Bradley et al., 1986; Yasui et al., 1988), no firm association with prognostic factors has been established. The aim of this study was to evaluate different methods of immunohistochemical identification of EGF receptors on human colonic cancers, and to investigate the relationship between receptor expression and morphological differentiation.

In all, 30 colonic carcinomas were studied, a portion being taken immediately after resection and placed into liquid nitrogen. Frozen sections (6 μm) were then cut, air dried overnight, wrapped in aluminium foil and stored at −20°C. The primary antibody used was mouse monoclonal anti-human EGF receptor (Amersham) which is a class G2b immunoglobulin produced using trypsinised A431 cells as the immunogen (Waterfield et al., 1982). This antibody recognises an antigenic determinant located on the extracellular domain of the receptor (Mayes & Waterfield, 1984) but does not compete with EGF for the ligand binding site (Gulllick et al., 1984).

In order to identify EGF receptors, three methods of immunoperoxidase staining were evaluated: indirect, peroxidase–antiperoxidase (PAP) and streptavidin–biotin (sABC) (Guesdon et al., 1979; Polak & Van Noorden, 1983; Sternberger, 1979). The indirect method utilised peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts) as the second stage. For PAP staining, rabbit anti-mouse peroxidase–conjugated rabbit anti-mouse was followed by peroxidase–mouse anti-peroxidase complex (Dakopatts). Both of these systems were tried using single and double techniques. The sABC method employed biotin-conjugated rabbit anti-mouse immunoglobulin and streptavidin complexed to biotinylated peroxidase (Dakopatts).

Various concentrations of all reagents were tested in order to obtain optimum staining, the primary antibody being used at 1:25, 1:50, 1:100, 1:200 and 1:400, and sections were incubated at room temperature for 60 min. The rabbit anti-mouse immunoglobulin was absorbed with 10% human AB serum. Bound peroxidase was visualised using the diaminobenzidine/H₂O₂ reaction, and sections were counterstained with haematoxylin, dehydrated and mounted in DPX. Two methods were used to block endogenous peroxidase staining. The first consisted of periodic acid at 0.228% placed on to the slides for 45 s after air drying (Kelly et al., 1987), and the second involved making up the secondary antibody in TBS containing glucose oxidase (1.5 u ml⁻¹) and glucose (0.2M) (Koller et al., 1986).

Frozen sections of human placental tissue were used as positive controls, and negative controls consisted of human thyroid tissue (Damjanov et al., 1986; Gusterson et al., 1979). In addition, every study slide was accompanied by a substitution control in which the primary antibody was replaced by non-immune mouse IgG (Sigma). At histological examination, the intensity of peroxidase staining was graded as 0, + or ++, and the colonic tumours were classified as well/moderately or poorly differentiated. This was done by two independent observers, and the grading of peroxidase staining intensity was performed without knowledge of the degree of differentiation.

In all cases, background and non-specific staining was minimal, although endogenous peroxidase in granulocytes was encountered when blocking was not employed. The syncytiotrophoblast of the placental tissue stained positively using all techniques, but strongest staining was obtained using the sABC method. No staining of formalin-fixed placental tissue could be obtained by any means. In the colonic tumours, positive staining was only obtained using the double PAP system, with peroxidase-conjugated rabbit anti-mouse as the secondary antibody, and the sABC method (Table I). Of these two, the sABC technique gave more intense, clearer staining and was much less time consuming. The ideal dilutions were found to be 1:100 for the primary antibody and 1:200 for the biotinylated rabbit anti-mouse. Twenty-seven out of the 30 colonic cancers stained for EGF receptor, and the staining was confined to the cytoplasm of the neoplastic epithelial cells within the tumour. Both the periodic acid and glucose oxidase methods were effective in blocking endogenous peroxidase, but periodic acid abolished all EGF receptor staining, even in placental tissue.

Table I Intensity of immunohistochemical staining for EGF receptor on human placenta and colonic carcinoma using a variety of methods

| Placenta | Colonic carcinoma |
|----------|-------------------|
| Single indirect | + | 0 |
| Double indirect | + + | 0 |
| Single PAP | + | 0 |
| Double PAP | + | 0 |
| Single PAP + PCRAM | + | 0 |
| Double PAP + PCRAM | + | 0 |
| sABC | + | + |

Results are based on the optimal primary antibody dilution of 1:100. 0, no staining; +, weak staining; + +, strong staining; PAP, peroxidase–antiperoxidase; PCRAM, peroxidase-conjugated rabbit anti-mouse; sABC, streptavidin–biotin complex.
The glucose oxidase technique did not affect the immunoreactivity of the EGF receptor on any of the sections. Intensity of staining was uniform throughout individual tumours, but it was significantly greater in the poorly differentiated tumours when compared with the well/moderately differentiated cancers (Table II).

In this study we have shown that EGF receptors can be identified on the neoplastic cells of invasive human colonic cancers using a well characterised commercially available mouse monoclonal antibody, and we found the sABC system to be the most suitable for this purpose. Less sensitive methods were inadequate, and this presumably relates to the low concentrations of receptor found in colonic carcinoma using radioiodide binding (Yasu et al., 1988).

EGF receptors have been demonstrated on a variety of human tissues (Damjanov et al., 1986; Gusterson et al., 1984) including squamous cell carcinoma (Ozanne et al., 1986), and neoplasms of the lung (Veale et al., 1987), breast (Sainsbury et al., 1985), bladder (Neale et al., 1985) and stomach (Yasu et al., 1988). In breast cancer, EGF receptor expression has been shown to be associated with an absence of oestrogen receptors, poor differentiation and increased risk of early recurrence and death (Sainsbury et al., 1985c, d, 1987, 1988). Similarly, in bladder cancer EGF receptor positive tumours tend to be invasive and poorly differentiated (Neale et al., 1985), and in stomach carcinoma early tumours are less likely to have receptors than advanced cancers (Yasu et al., 1988).

Two previous studies have demonstrated EGF receptors in colonic carcinoma (Bradley et al., 1986; Yasui et al., 1988). In the first of these, radioligand binding, Western transfer and indirect immunofluorescence were used, and moderately well differentiated tumour cell lines were found to express receptor activity to a greater extent than poorly differentiated lines. There was, however, no detailed information on naturally occurring tumours in this report (Bradley et al., 1986). In the second study, no association between tumour grade and EGF receptor expression could be demonstrated, and only 77% of the tumours were receptor positive (Yasu et al., 1988).

Clearly, there are contradictory findings regarding the detection and significance of EGF receptors on human tumours. This may indicate biological differences between different tumour types, but it may also be related to the use of antibodies which recognise different epitopes on the receptor. Our data suggest that, when a highly sensitive technique is used, the majority of colonic carcinomas can be shown to express EGF receptors and that there is an association between the degree of expression and histological differentiation. Further elucidation of the prognostic significance of this finding must await follow-up of larger numbers of patients.

This work was supported by grants from the Royal College of Surgeons of Edinburgh and the Grampian Health Board.

### Table II

| Staining intensity | 0/+ | ++ |
|--------------------|-----|----|
| Moderately/well differentiated | 16  | 7  |
| Poorly differentiated       | 0   | 7  |

$\chi^2 = 7.83$ (With Yates' correction); $P<0.01$. Difference in percentage of tumours exhibiting + + staining = 69%, with a 95% confidence interval of 50–88%.

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