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Expression of the C-terminal peptide of human pro-bombesin in 361 lung endocrine tumours, a reliable marker and possible prognostic indicator for small cell carcinoma

Q.A. Hamid [1], B.J. Addis [3], D.R. Springall [1], N.B.N. Ibrahim [4], M.A. Ghatei [2], S.R. Bloom [2], and J.M. Polak [1]

[1] Departments of Histochemistry and [2] Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 OHS, UK

[3] Department of Histopathology, Brompton Hospital, London SW3 6HP and [4] Department of Histopathology, Frenchay Hospital, Bristol BS16 1LE UK

Summary. Small cell carcinoma of the lung is a highly malignant tumour. Its known biological products which include bombesin, do not allow the prediction of tumour behaviour. Molecular biology has revealed the amino acid sequence of human pro-bombesin, which consists of a signal peptide, the bioactive bombesin molecule and a C-terminal peptide. We have raised a rabbit antiserum to the first (N-terminal) 21 amino acids of the predicted C-terminal peptide. A total of 505 (361 neuroendocrine) surgically resected pulmonary tumours were evaluated for the presence of immunoreactive bombesin and C-terminal peptide. Strong immunostaining was obtained with the antiserum to the C-terminal peptide of human pro-bombesin in 70% of the small cell carcinomas (175/250), in 63% of atypical (aggressive) carcinoids (31/49) but only in 16% of benign carcinoids (10/62). In contrast, bombesin immunostaining was focal and only moderately strong and the relative proportion of positive cases was quite evenly distributed amongst the neuroendocrine tumours: 35% of carcinoids (22/62), 22% of atypical carcinoids (11/49) and 25% of small cell carcinoma (62/250). None of the squamous, adeno, or large cell undifferentiated carcinomas were immunoreactive for bombesin or the C-terminal peptide. Radioimmunoassay and chromatography of extracts of tumours recovered from wax blocks revealed high concentrations of C-terminal peptide immunoreactivity (241 ± 66 pmol/g of tissue) in all 12 small cell carcinomas studied, moderate concentrations in carcinoid tumours (50 ± 7 pmol/g) and none in non-small cell carcinomas. Patients with tumours showing immunoreactivity to the C-terminal peptide of human pro-bombesin had a significantly shorter survival time than those without immunoreactive peptide (185 ± 16.49 days, mean $\pm$ SEM, and with 1128 ± 226 days, respectively $P > 0.02$). The apparent presence of the C-terminal peptide of human pro-bombesin in higher concentrations than bombesin in the more malignant class of endocrine tumours, mainly small cell carcinomas associated with the poorest prognosis, suggests that the antiserum to this C-terminal peptide is not only a useful pathological marker but may prove to be of value in investigating the biological behaviour of small cell carcinomas and predicting the clinical course of the disease.

Key words: Bombesin – Human pro-bombesin – Lung – Small cell carcinoma

Offprint requests to: J.M. Polak at the above address

Introduction

In the last 50 years there has been an alarming increase in the incidence of bronchial carcinomas, (Miller 1980) of which small cell carcinoma comprise 20–25% (Hardly et al. 1981). Small cell carcinoma is an aggressive neoplasm, having the poorest prognosis of all lung tumours with an overall 5-year survival rate of only 2%. (Carter 1983). These tumours are generally sensitive to chemotherapy and radiation which emphasises the importance of accurate, early diagnosis (Cohen and Matthews 1978). However, they are very heterogenous in nature (Gazdar 1980) and some of them respond poorly to such non-surgical treatment (Minna et al. 1982; Miller et al. 1983). Histological features are of little value in predicting the clinical course of the disease (Carney et al. 1980) and there are no known biological indicators which can explain the behavioural heterogeneity (Vincent 1982).

Small cell carcinoma has been characterised as

a neuroendocrine tumour by the presence of typical neurosecretory granules and its immunoreactivity to a number of general markers for neuroendocrine differentiation, (Gould and Chefjec 1978; Blobel et al. 1985) including neuron-specific enolase (Sheppard et al. 1984) and protein gene product 9.5 (Dhillon et al. 1985). However, immunostaining of these antigens does not allow predictions to be made on the biological behaviour of a given tumour.

Many hormones have been shown to be produced by small cell carcinomas, including adrenocorticotrophic hormone, growth hormone, antidiuretic hormone, calcitonin and bombesin. (Sorenson et al. 1981; Warren et al. 1984; Moody et al. 1981). Bombesin is a 14 amino acid peptide first isolated from the skin of a European amphibian (Anastasi et al. 1971). Its mammalian analogue is gastrin-releasing peptide (GRP or human bombesin), (McDonald et al. 1979) a 27 amino acid peptide present in the nervous system, (Walsh et al. 1979) the gastrointestinal tract (Polak et al. 1976) and the pulmonary tract (Yamaguchi et al. 1982). In the latter, it is present in the pulmonary neuroendocrine cells, which are most prevalent during the neonatal period (Wharton et al. 1978). It is also produced by neuroendocrine tumours (Price et al. 1985). Bombesin causes various proliferative responses, (Rozengurt and Sinnett-Smith 1983; Willey et al. 1984) including stimulation of the clonal growth and DNA synsthesis of small cell carcinoma in vitro (Weber et al. 1985). Monoclonal antibodies to bombesin block the binding of hormone to cellular receptors and inhibit the clonal growth of small cell carcinoma in vitro and the growth of small cell carcinoma xenografts in vivo (Cuttitta et al. 1985).

Although many of cell lines derived from small cell carcinomas contain extractable bombesin, it is difficult to localise the peptide immunocytochemically (Gould et al. 1983a) and only 5% of patients with small cell carcinoma have elevated plasma levels of bombesin (Pert and Schumacher 1982). This has limited the value of bombesin as a morphological and biological marker for these tumours.

Using RNA from a pulmonary endocrine tumour, Spindel et al. obtained cDNA clones for the prepro-human bombesin (prepro-gastrin-releasing peptide, GRP) gene (Spindel et al. 1984), which encodes a putative pro-GRP molecule consisting of a signal sequence, GRP itself and a GRP-associated peptide (C-terminal peptide), the function of which is not yet known (Fig. 1). Subsequently, the same authors found two types of prepro-GRP

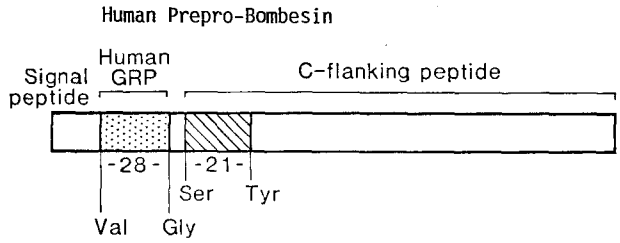


Fig. 1. Diagrammatic representation of the human pro-bombesin. The region of the C-terminal peptide in which the antibody has been raised is shaded.

cDNA clones in a cDNA library from pulmonary carcinoid tumour tissue, which differ in the presence of a 19 base insertion in the GRP-associated peptide occuring after amino acid 98 in pro-GRP (Spindel et al. 1986). Recently, Sauville et al. (1986) have found three types of prepro-GRP mRNA which differ in the structure of the C-terminal flanking peptide. These three mRNAs represent different transcriptional products from a single gene.

This study was performed to determine whether the C-terminal flanking peptide of human pro-bombesin is expressed in lung endocrine tumours for three reasons. Firstly in view of evidence for the production of abnormal molecular forms of hormones from their precursor molecules in tumour cells (Holst 1983). Secondly our recent observation that immunoreactive C-terminal peptide of human pro-bombesin occurs in extra-pulmonary small cell carcinoma (Springall et al. 1986). Thirdly to determine whether the C-terminal peptide of human bombesin is expressed in lung endocrine tumours and if so to assess its possible role as a morphological marker and to evaluate its usefulness in predicting the biological behaviour of these tumours.

Materials and methods

Tissue preparation. A total of 505 lung tumours was investigated. The material consisted of surgical resection specimens, in the form of paraffin blocks of formalin fixed tissue, some dating back to 1969.

Sections (5 μm thick) from all blocks were taken up on poly-L-lysine-coated slides (Huang et al. 1983), air-dried at 37° C and de-waxed in xylene and rehydrated for routine histology and immunocytochemistry.

Histological classification. The lung tumour sections were stained with haematoxylin and eosin to assess the tumour cell type.

Antibodies. Details of the primary antisera used are shown in Table 1.

The antibody to the C-terminal peptide of human pro-bombesin was raised in rabbits against a synthetic fragment

Table 1. Details of the antisera used

Antiserum to	Type	PAP dilution	Absorption[a] (n mol/ml)
Bombesin	rabbit polyclonal	1/2000	1.0
C-terminal peptide of human pro-bombesin	rabbit polyclonal	1/8000	1.0

[a] Concentration (n mol of homologous antigen/ml of optimally diluted antibody) required to abolish immunostaining by the PAP method

consisting of the N-terminal 21 amino acids of the predicted sequence of the C-terminal peptide. For immunisation, the peptide was coupled to bovine serum albumin using ethyl carbodiimide.

Immunohistochemistry. Sections were immunostained using the peroxidase anti-peroxidase (PAP) method (Sternberger et al. 1970). After de-waxing, endogenous peroxidase activity and nonspecific staining due to the bridging antisera were blocked by sequential incubation for 30 min in 0.03% hydrogen peroxide and 30 min in normal goat serum (1:30). The primary antibodies (Table 1) were applied and incubated for 16–20 h at 4° C. After thorough rinsing in buffer, the sections were incubated with an excess of goat anti-rabbit immunoglobulin G (Miles Labs, USA). After further rinsing in buffer, the sections were incubated with PAP complex (Miles Labs., dilution 1:500). Visualisation of the PAP complex was achieved by the diaminobenzidine method of Graham and Karnovsky (Graham and Karnovsky 1966).

Radioimmunoassay. Radioimmunoassays were performed on tissue taken from paraffin blocks of tumours from 20 cases. These were 12 small cell carcinomas, 4 carcinoids and 4 non-small cell carcinomas. The blocks were dewaxed in xylene for 16 h, and then passed through different graded alcohols (absolute, 90%, 70%, 50% and 30% aqueous solutions, 2 h in each). The tissues were then extracted in 0.5 M acetic acid at 100° C for 10 min. Extracts were assayed for bombesin and the C-terminal peptide of human pro-bombesin using the same antisera as were used for immunocytochemistry.

Tumour extracts were gel filtered on a column (1.5 × 100 cm) packed with Sephadex G-50 superfine and eluted at a flow rate of 3 ml/h at 4° C with 0.06 M phosphate buffer, PH 7.4, containing 0.2 M sodium chloride, and 3 mg/ml BSA. The column was precalibrated with dextran blue 2000 (mol wt, 2×10^6), cytochrome C (mol wt, 12,384) and $Na^{125}I$ as a bed volume marker (Ghatei 1982). The concentrations of immunoreactive peptide were expressed as the mean ± SEM. The elution co-efficients for each immunoreactive peak are calculated according to the method of Laurent and Killander (1964).

Follow up. The files of the 200 patients with small cell carcinoma were reviewed to determine the clinical course of the disease and the survival of the patients following surgical resection of the tumour. We chose only patients who did not die post-operatively and did not receive any sort of chemotherapy or radiotherapy, thus 109 cases were available for correlation of the C-terminal peptide staining and survival rates. The data was statistically assessed by Mann-Cohieney test.

Table 2. Immunocytochemistry results of lung tumours

Type of tumour	Total number	Immuno-reactive for bombesin	Immuno-reactive for the C-terminal peptide of human pro-bombesin
Small cell carcinoma	250	62	175
– oat cell type	97	22	65
– intermediate cell type	153	40	110
Atypical carcinoid	49	11	31
Benign carcinoid	62	22	10
Squamous cell	65	0	0
Adenocarcinoma	57	0	0
Large cell undifferentiated carcinoma	22	0	0

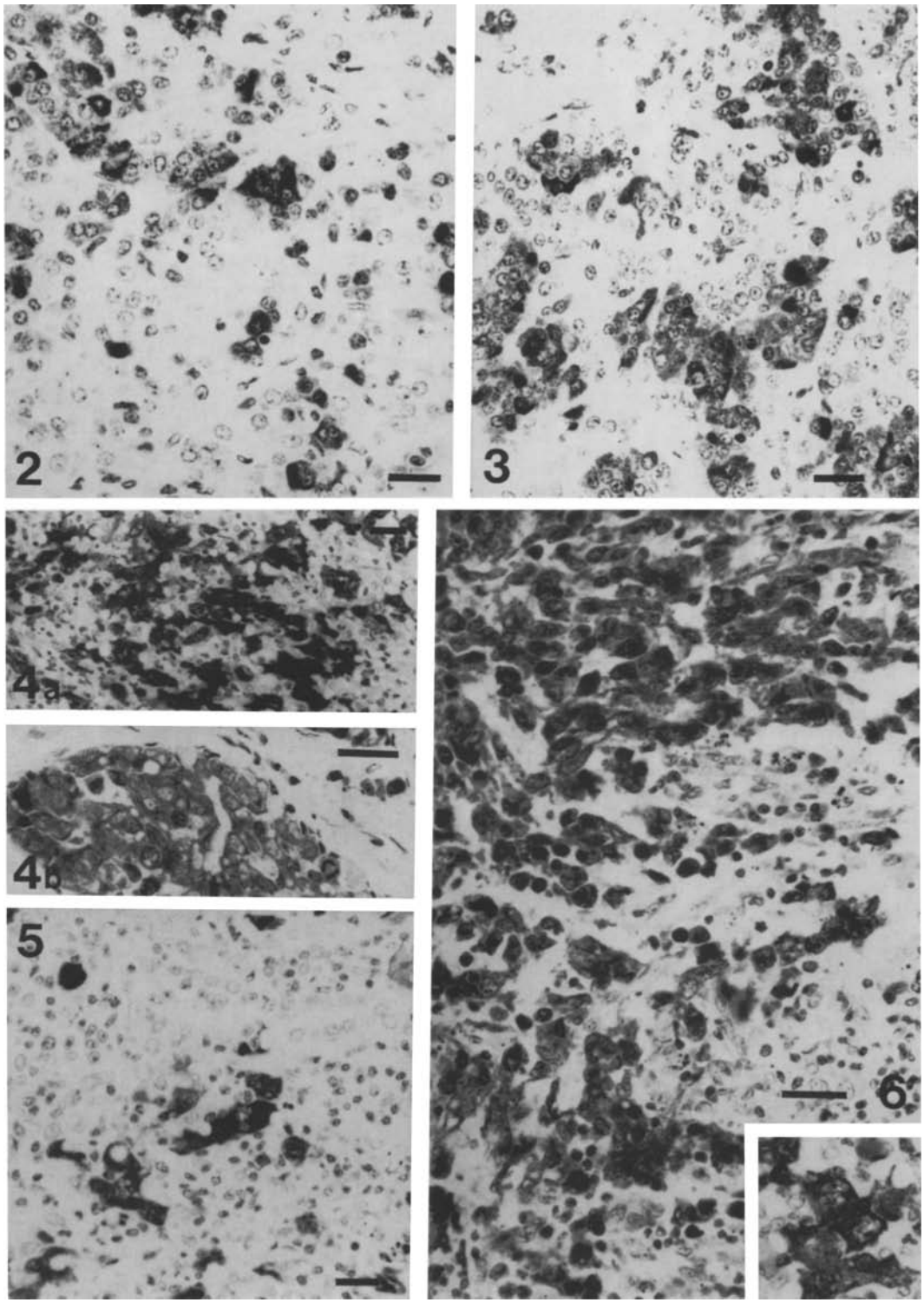
Results

Histological classification

The 505 lung tumours (361 endocrine) were classified as small cell carcinoma ($n=250$), carcinoids ($n=62$), atypical carcinoids ($n=49$), squamous cell carcinoma ($n=65$), large cell carcinoma ($n=22$) and adenocarcinoma ($n=57$). The small cell carcinomas were further divided into oat cell ($n=97$) and intermediate cell ($n=153$) types.

Immunocytochemistry

The results of immunohistochemical staining are summarised in Table 2. Bombesin-like immunoreactivity was demonstrated in 35% of carcinoids, 22% of atypical carcinoids and 25% of small cell carcinomas (Figs. 2 and 5). The immunoreactivity was focal and moderately strong in most of the tumour cases. In contrast, antibodies to the C-terminal peptide of human probombesin, strong and diffuse immunostaining was detected in 70% of small cell carcinomas, 63% of atypical carcinoids and only 16% of carcinoid tumours (Figs. 3, 4 and 6). None of the squamous, large cell or adenocarcinomas were immunoreactive for either bombesin or the C-terminal peptide of human pro-bombesin. Immunoreactivity was abolished following absorption of each antiserum with its homologous antigen (Table 1).



Figs. 2, 3. Carcinoid tumour of the lung, showing focal immunostaining with antiserum to bombesin **2** and more diffuse immunostaining with antiserum to C-terminal peptide of human pro-bombesin **3** PAP, counterstained with haematoxylin ×400. NB bar = 20 μm

Figs. 4a, b. Atypical carcinoid tumour of the lung showing strong **a** and diffuse **b** immunostaining with antiserum to C-terminal peptide of human pro-bombesin. PAP, counterstained with haematoxylin. **a** ×200; **b** ×480. NB bar = 20 μm

Fig. 5. Focal bombesin immunoreactivity in small cell carcinoma of the lung PAP ×320. NB bar = 20 μm

Fig. 6. Small cell carcinoma of the lung showing strong and diffuse immunostaining with antiserum to C-terminal peptide of human pro-bombesin. Some cells show nuclear staining. PAP, counterstained with haematoxylin ×480. NB bar = 20 μm

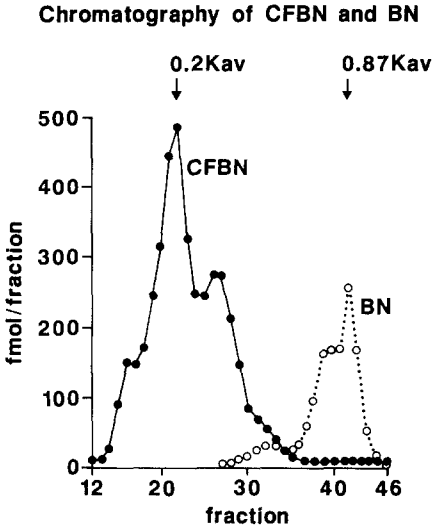


Fig. 7. Chromatographic profile (Sephadex G50) of extractable bombesin (*BN*) and C-terminal flanking peptide (*CFBN*) immunoreactivity in a small cell carcinoma of the lung recovered from paraffin wax

Radioimmunoassay results

Considerable concentrations of extractable C-terminal peptide of human pro-bombesin were found in small cell carcinomas (241 ± 66 pmol/g, mean $\pm$ SEM, $n=12$). The levels were lower in carcinoid tumours (50 ± 7 pmol/g) while in non-small cell carcinomas (adeno, large cell undifferentiated and squamous) the C-terminal peptide of human pro-bombesin was undetectable.

Gel-permeation chromatography of extracts of small cell carcinoma revealed a major peak immunoreactive for the C-terminal flanking peptide of a $k\hat{a}v = 0.18$ which eluted between the dextran blue and cytochrome C (Fig. 7). The majority of bombesin-like immunoreactivity eluted at the same $k\hat{a}v$ (0.82) as the C-terminal decapeptide of gastrin releasing peptide, with a smaller fraction at the same position ($k\hat{a}v = 0.56$) as whole gastrin releasing peptide molecule.

Correlation of survival time with immunocytochemistry

Table 3 shows the correlation of the survival times of 109 patients with small cell carcinoma immunoreactive for the C-terminal peptide of human pro-bombesin. The survival time of patients whose tumours were independently (blind) assessed to be immunoreactive to the C-terminal peptide of human pro-bombesin was 185 ± 16.49 days (mean $\pm$ SEM), while those whose tumours did not show significant immunoreactivity had a mean survival time of 1128.15 days ± 226.6. The difference in the survival of the two groups of patients was statistically significant ($P > 0.02$).

Table 3. Correlation of the immunoreactivity for the C-terminal peptide of human bombesin with survival of 109 patients with small cell carcinoma

Number of patients	Imunostaining with antisera to C-TPBN	Mean survival following the operation (days)	Standard error of mean (SEM)
71	positive	185*	16.49
38	negative	1128.1*	226
Total 109			

* Statistically significant difference $P > 0.02$

Discussion

This study describes the expression of bombesin and the C-terminal peptide of human pro-bombesin in 361 surgically resected lung neuroendocrine tumours.

The search for specific biological products which are possible indicators of the behaviour of small cell carcinomas has so far been disappointing (Hansen 1980). Although bombesin is an interesting example of a hormone produced by small cell carcinomas, which seems to have an autocrine growth effect, (Gould et al. 1983b) it does not appear to be a useful marker morphologically or clinically (Gould et al. 1983a). In our series, only 62 cases of the 250 small cell carcinomas investigated were immunoreactive to bombesin antiserum. The lack of immunoreactivity could be due to the scarcity of secretory granules in this type of tumour, or to the tumour cells producing molecular forms of the active hormone from the precursor molecule that are not recognised by the antibody.

Most, if not all, of the known active peptides, are derived from larger molecule (prohormones), and the primary mechanism of their processing involves proteolytic cleavage at sites characterised by the presence of consecutive basic residues (Martinez and Potier 1986). In many cases, a single, large polypeptide prohormone has been found to serve as precursor for several biologically active peptides (Lazure 1983). The pro-bombesin molecule consists of a signal peptide, the bioactive bombesin molecule, and a large C-terminal flanking peptide of variable amino acid sequence at its C-terminal. The antiserum used in this study was raised against a 21 amino-acid fragment of this latter peptide (Fig. 1). This fragment is in the con-

served part of the peptide and antisera to it should therefore recognise all known different forms of the C-terminal peptide of human pro-bombesin. Using antisera to this fragment, 70% (175/250) of small cell carcinomas were immunostained compared with only 16% (10/62) of carcinoid tumours. C-terminal peptide immunoreactivity was demonstrated in 63% (31/49) of atypical carcinoids, which behave in a more aggressive manner than carcinoids.

Thus, while bombesin immunoreactivity has a more or less similar distribution in carcinoids, atypical carcinoids and small cell carcinomas, the C-terminal peptide of human pro-bombesin is detectable in a greater proportion of cases of the more malignant tumours (small cell carcinoma and atypical carcinoid) (Table 2).

Similar results were reported in extrapulmonary small cell carcinoma by Springall et al. (1986). Recently, other groups have detected the C-terminal peptide of human pro-bombesin in small cell carcinoma cell lines by radioimmunoassay using an antibody to the C terminal part of the C-terminal peptide of human pro-bombesin (Reeve et al. 1986). The apparent increase in detectable immunoreactivity to bombesin gene products in small cell carcinomas fits well with the proposed autocrine hypothesis which suggests that a cell can produce and secrete a hormone-like substance which interacts with specific membrane receptors to induce proliferation (Sporn and Todaro 1980). Thus a role for pro-bombesin derivatives as putative growth promoting factors may be a possibility. This suggestion is supported by our findings of a lower survival rate in patients whose tumours were immunoreactive for the C-terminal peptide of human pro-brombesin rather than bombesin. Recently, binding of radio-labelled C-terminal peptide of human pro-bombesin to cultured small cell carcinoma cells has been demonstrated (Reeve 1986).

None of the squamous, large cell or adenocarcinomas were immunoreactive for the C-terminal peptide of human pro-bombesin, thus emphasising the benefit of using this antiserum not only as a biological indicator but also as a good morphological marker.

As it is difficult to get fresh samples of small cell carcinoma, radioimmunoassay was performed on extracts of tissue processed to paraffin blocks. Despite this adverse treatment high concentrations of the C-terminal peptide were detected in these tumours. These levels probably grossly underestimate those present in vivo, because it is likely that in the fixation process only a small quantity of soluble bombesin gene products was trapped and thus rendered available for extraction, assay and chromatography. Furthermore, the particular form surviving processing may not necessarily have been representative of the peptide(s) originally synthesised. It is fortunate that sufficient detectable bombesin gene peptide products remained in the extracts to allow confirmation of their presence by specific radioimmunoassay and chromatography.

The high expression of the C-terminal peptide of human pro-bombesin in a large number of small cell carcinomas, which are known generally to be poorly granulated, indicates a possible alternative pathway from peptide synthesis to release. This is supported by our own preliminary electron immunocytochemical data showing a granular and in particular cytoplasmic localisation of the C-flanking peptide in cultures of small cell carcinomas. It would thus seem justifiable to speculate that peptides could be produced and voided by a cell, particularly a transformed or otherwise compromised cell, without packaging into storage granules. Some evidence does exist for such a mechanism in other systems (Roth et al. 1985). In the case of the C-terminal peptide of human pro-bombesin, our antiserum recognises the N-terminal sequence. Thus in theory bombesin and the C-terminal peptide of human pro-bombesin should be dissociated before this antiserum can react. However, there may be amino acid sequences in both the C-terminal peptide of human pro-bombesin and other portions of pro-bombesin which can react with the polyclonal anti-C-terminal peptide of human pro-bombesin serum. If the antiserum is in fact specific for the free N-terminal region of the C-terminal peptide of human pro-bombesin our results suggest that the prohormone is cleaved in such a way that bombesin is stored in granules, whereas the C-terminal peptide of human pro-bombesin exists in addition extra-granular areas of the cytoplasm.

In conclusion, we have shown that the C-terminal peptide of human pro-bombesin is found in all types of endocrine tumours of the lung, but is detectable predominantly in highly malignant small cell carcinomas associated with poor survival rates, and less frequently, in benign carcinoid tumours. In small cell carcinomas, this peptide is therefore not only a useful diagnostic indicator, but may also prove to be of value in determining prognosis and the best mode of therapy of individual cases.

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