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Analysis of tumour necrosis factor *a*-specific, lactose-specific and mistletoe lectin-specific binding sites in human lung carcinomas by labelled ligands

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Summary. Receptor sites can be visualized by labelled ligands as an alternative to receptor-specific antibodies, as substantiated for two different receptor classes. Recombinant tumour necrosis factor α (TNF) was biotinylated via amino-groups and the resultant probe was applied to formalin-fixed, paraffin-embedded tissue sections of 94 primary bronchial carcinomas and to normal peripheral lung parenchyma. In addition, monoclonal antibodies specific for neuron-specific enolase (NSE) and TNF itself were used. The biotinylated β-galactoside-specific mistletoe lectin, which exhibits dose-dependent immunomodulatory and toxic potency, and two probes that specifically detect certain types of sugar receptors were employed to illustrate further the feasibility of using ligands for receptor localisation. The tumours comprised 62 small cell lung carcinomas, 10 epidermoid carcinomas, 11 adenocarcinomas and 11 large cell anaplastic carcinomas. Expression of TNF-binding sites was found in 39 of the small cell lung carcinomas and in 13 of the non-small cell lung carcinomas. Binding capacity for the TNF-specific antibody was seen in similar proportions of small cell lung carcinomas and of non-small cell lung carcinomas. None of the normal lung parenchymas revealed significant staining. Binding capacities to mistletoe lectin were seen in all normal lung parenchymas and in nearly all cases of adenocarcinoma (10/11). A correlation between the expression of NSE and the binding capacities to TNF was detected. Endogenous lectins, specific for lactose or β-GalNAc, were displayed in nearly one half of the small cell lung carcinoma cases (44% or 45% respectively) and in about 25% of the non-small cell lung carcinoma cases.

Key words: Tumour necrosis factor α – Human lung carcinoma – Neuron-specific enolase – Endogenous lectins – Mistletoe lectin

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Introduction

Carrier-immobilized ligands or labelled ligands that exert receptor-binding specifity are a reasonable alternative to antibodies for receptor localization. This principle has already taken advantage of using localization of endogenous sugar receptors by neoglycoproteins (Gabius 1988, 1991; Gabius et al. 1991a; Kayser et al. 1989a, b, 1991). Similarly, growth factors or hormones have been labelled to generate tools for this purpose (Kayser et al. 1990a, b, c). It is, therefore, reasonable to address the question of whether a biotinylated cytokine enables specific visualization of receptor sites in tissue sections. Moreover, it is interesting to monitor the concomitant presence of the cytokine by an antibody. The present study was performed to increase our knowledge about binding capacities of human lung carcinoma cells to tumour necrosis factor α (TNF) and to carbohydrate-exposing neoglycoproteins. The choice of two ligand classes is intended to illustrate a more generalized applicability of this approach. In addition to the histochemical application of the labelled ligands the relationship of binding to the expression of tumour-associated proteins, especially to neuroendocrine markers such as neuron-specific enolase (NSE), to TNF and to mistletoe lectin is evaluated. This lectin binds β-galactoside-containing glycoconjugates and is clinically of special interest due to its immunomodulatory potency by enhancement of TNF secretion (Gabius et al. 1991b).

TNF belongs to the family of cytokines and is involved via receptor-mediated processes in regulatory functions of inflammation and cellular immunity (Mc Call et al. 1989; Brockhaus et al. 1990; Smith and Baglioni 1989; Vilcek and Lee 1991). It was, therefore, especially tempting to elucidate whether biotinylated TNF is effective in localizing its binding sites in fixed tissue specimens. So far, biotinylated TNF has been shown to recognize specific receptor sites in native cells effectively (Ranges et al. 1989; Schall et al. 1990).

We selected lung tumours for this study. In human lung carcinomas two main cell types can be distin-

guished: small cell lung carcinomas which usually respond favourably (although for a limited time) to cytostatic drug regimens, and non-small cell lung carcinomas. In geneal, their growth is not reduced by cytostatic therapy, which encouraged us to examine the influence of immunomodulatory agents such as mistletoe lectin on human lung carcinoma growth. Because of the supposed importance of protein-carbohydrate interactions for cellular communication and of TNF in lectin-mediated anti-tumoral effects (Gabius 1991), respective receptor sites were determined in the lung cancer types.

The two major groups of lung carcinomas show some similarities in respect of their histomorphological appearance: small cell lung carcinomas and, less frequently, large cell lung carcinomas usually grow without dense inflammatory infiltrates in contrast to epidermoid carcinomas and adenocarcinomas (Kayser et al. 1986). Small cell lung carcinomas and large cell carcinomas usually contain large areas of infarct-like necrosis. Small cell carcinomas and adenocarcinomas usually show multiple micrometastases in close connection with the boundary of the tumour mass. These findings encouraged us to monitor cellular determinants such as receptors for TNF, carbohydrates or immunomodulatory agents to uncover histochemical differences.

Materials and methods

Commercially available recombinant tumor necrosis factor α (kindly provided by Eurocetus, Amsterdam) was biotinylated with biotinyl-N-hydroxysucchinimide ester, as described for neoglycoproteins or epidermal growth factor (Kayser et al. 1989a, 1990a, b). β-N-acetyl-D-galactosamine (β-GalNAc) and lactose were chemically attached to bovine serum albumin and the product was biotinylated, as described (Kayser et al. 1989a). The toxic and immunomodulatory β-galactoside-specific lectin from mistletoe (ML-1) was purified as previously described (Gabius 1990) and biotinylated under activity-preserving conditions as reported recently (Gabius et al. 1991a). The monoclonal antibodies against TNF and the gamma subset of the NSE were obtained from Boehringer, Mannheim, FRG and Biotrend, Köln, FRG, respectively. The biotinylated probes and the monoclonal antibodies were applied to 4–6 µm thick histological sections of formalin-fixed (buffered formalin, pH 6.9–7.3), paraffin-embedded tissues. The sections were incubated in 0.1% methanolic hydrogen peroxide for 30 min after deparaffination and rehydration. The non-specific background staining caused by protein-protein interactions was minimized by treatment with 1% carbohydrate-free bovine serum albumin (BSA) in TRIS-buffered saline after equilibration with 0.1 M TRIS buffer (pH 7.4). The sections were incubated with the biotinylated probes and with the monoclonal antibodies at a concentration 10 µg/ml at room temperature for 60 min in TRIS buffer. Biotinylated TNF was also applied in the presence of ovalbumin to reduce any binding to high-mannose glycopeptides, described for its native and denaturated form (Moonen et al. 1988; Muchmore et al. 1990). After thorough washin in 0.1 M TRIS buffer (pH 7.4) positive reaction of the biotinylated probes and monoclonal antibodies was visualized by application of the avidin-biotin complex (Camon, Wiesbaden) or a second biotinylated antibody and the development of the chromogenic product from the substrates diaminobenzidine and hydrogen peroxide. Finally, the sections were counterstained with haematoxylin and mounted. The sections comprised biopsy and surgical specimens of 62 small cell lung carcinomas, 10 epidermoid carcinomas, 11 adenocarcinomas, 11 large cell anaplastic carcinomas and 10 cases with normal lung parenchyma. The cell type of the cases was classified according to the rules of the WHO. Positive and negative controls were performed as usual (Kayser et al. 1990a). The specificity of the binding to the labelled ligands was controlled by competition reactions with the unlabelled ligands (ratio 1:50, labelled:unlabelled ligand). Further controls included the application of labelled carbohydrate-free carrier protein (BSA) with and without added unlabelled TNF, running the protocol under identical histochemical conditions. Cases were considered positive only if dark brown staining was observed in all the tumour cells or lung parenchymal cells or at least in clusters of them.

Results

The staining behaviour of the probes applied for different lung carcinomas and normal lung parenchyma is summarized in Table 1. As seen in a previous study (Kayser et al. 1990c) 60–70% of the small cell lung carcinomas and 10–20% of the non-small cell lung carcinomas showed positive staining with the NSE-specific antibody. Lactose-specific binding capacities were seen in 35–45% of the undifferentiated or anaplastic carcinomas (small cell and large cell carcinomas) and in only 15–25% of the more differentiated tumours (adenocarcinomas and epidermoid carcinomas). No major differences between the different cell types were evaluated for the staining of the β-GalNAc-specific probe. The mistletoe lectin-specific binding sites were observed in all cases with normal lung parenchyma and in a high percentage of the tumour cases. Presence of β-galactoside-containing glycoconjugates in addition to potential receptor sites, localized by the neoglycoprotein, is thus ascertained. The staining was usually strong and homogeneous. Notably, biotinylated TNF bound specifically to the sections. TNF-binding sites, detected by the biotiny-

Table 1. Expression of receptors specific for tumour necrosis factor α (TNF), lactose (Lac), mistletoe lectin (ML-1) and (β-GalNAc) as well as expression of neuron-specific enolase (NSE) and TNF in primary lung carcinomas (%)

Cell type	Probe applied					
	TNF-Bio	TNF-Ab	NSE	Lac	ML-1	β-GalNAc
Normal lung ($n=10$)	0	0	0	0	100	10
Small cell carcinoma ($n=62$)	63*	73	63*	44	56	45
Epidermoid carcinoma ($n=10$)	60	30	20	20	50	20
Adenocarcinoma ($n=11$)	45	45	18*	18	91	36
Large cell carcinoma ($n=11$)	18*	36	18*	36	64	18

TNF-Bio, biotinylated TNF; TNF-Ab, antibody against TNF
* Statistically significant ($p<0.05$, chi-square test)

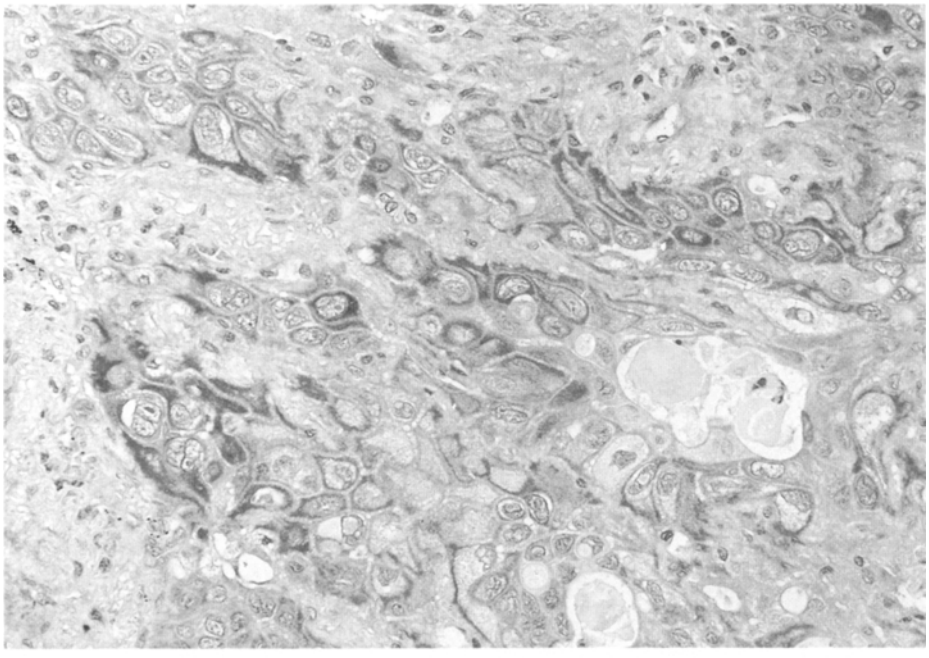

Fig. 1. Microphotograph of a small cell lung carcinoma showing positive staining for biotinylated TNF. PAP, ×420

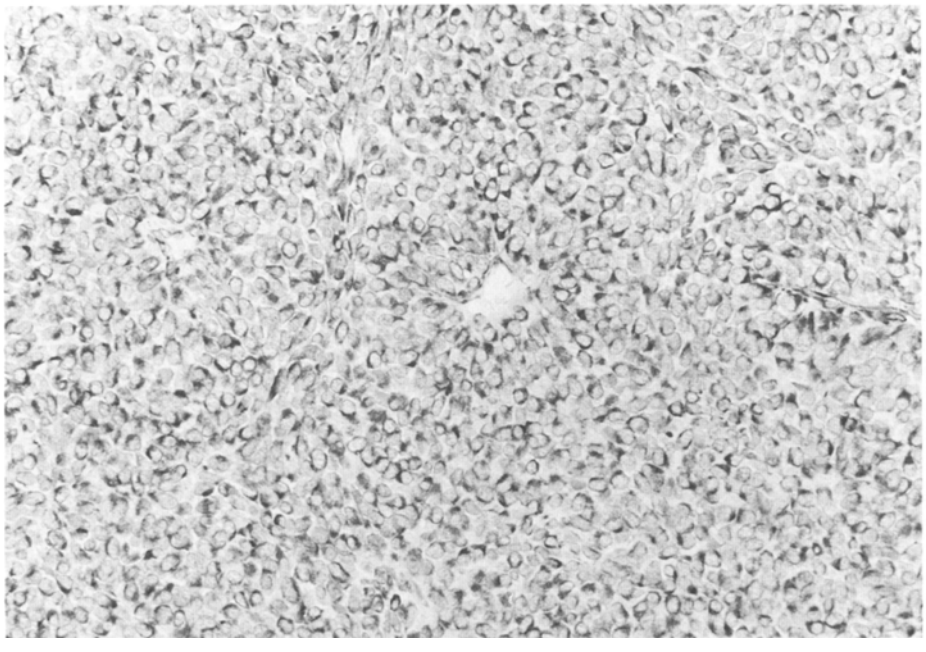

Fig. 2. Microphotograph of a small cell lung carcinoma showing positive staining for TNF-specific antibody. PAP, ×280

lated cytokine, were seen in the cytoplasm but seldom in the nucleus (Fig. 1). Presence of ovalbumin did not affect binding, excluding any contribution of carbohydrate recognition by TNF to the extent of observed staining. TNF itself was usually present in the cytoplasm, as shown with the antibody (Fig. 2). The staining with the biotinylated ML-1 was intense, as shown in Fig. 3. In view of the lectin-dependent increase of secretion of cytokines by mononuclear cells it is interesting to note that intra-tumour mononuclear inflammatory cells with strong positive staining to ML-1 were often observed.

The coexpression of NSE, detected immunohistochemically, in relation to the other probes applied is given in Table 2. About 80% of NSE-positive tumours reacted positively to the TNF antibody, compared with only 45% of the NSE-negative tumours. No significant differences could be observed with respect to ML-1, and the β-GalNAc and Lac-carrying probes.

A high proportion of carcinomas with demonstrable TNF-binding sites appeared to show intra-tumour TNF (Table 3). However, nearly 50% of the tumours without detectable TNF-binding sites appeared to express TNF. The percentages of the obtained coexpression to the

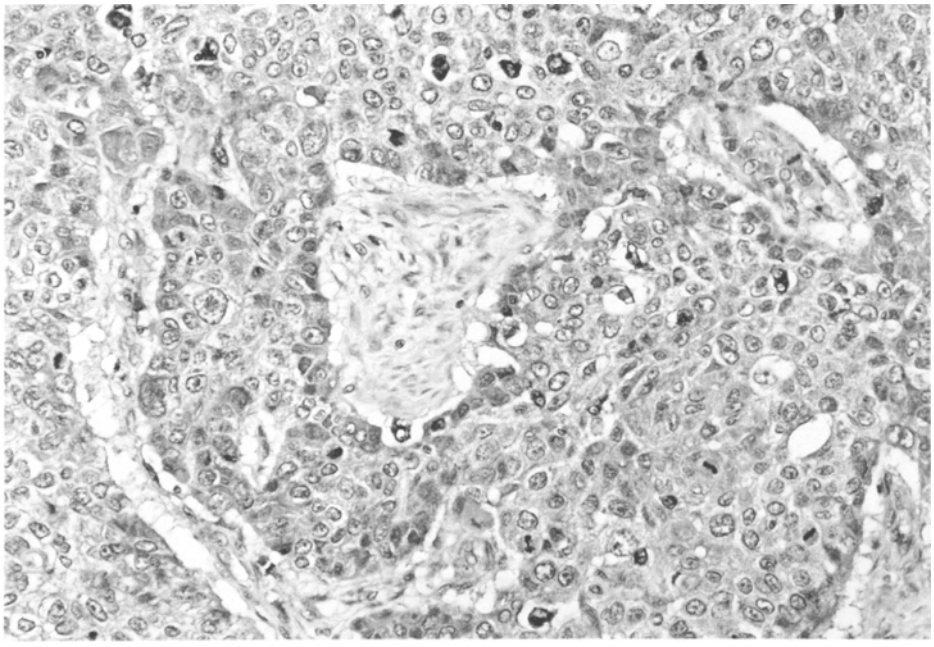

Fig. 3. Microphotograph of a small cell lung carcinoma showing positive staining for biotinylated mistletoe lectin. PAP, ×280

Table 2. Coexpression of NSE in relation to the expression of TNF and the receptors for TNF, Lac, ML-1 and β-GalNAc (%)

	Positive to				
	Lac	β-GalNAc	ML-1	TNF-Bio	TNF-Ab
NSE positive ($n=45$)	44	49	58	69	78
NSE negative ($n=49$)	31	29	63	43	45

Table 3. Coexpression of TNF-specific receptors in relation to the expression of NSE, TNF and to the expression of receptors specific for Lac, ML-1 and β-GalNAc (%)

	Positive to				
	Lac	β-GalNAc	ML-1	NSE	TNF-Ab
TNF-receptor positive ($n=52$)	58	60	65	60	73
TNF-receptor negative ($n=42$)	12	12	48	24	45

other structures, detectable with the applied probes, are given in Table 3. No differences could be observed between small cell lung carcinomas and non-small cell lung carcinomas.

Discussion

TNF belongs to the family of cytokines and is a polypeptide mediator that induces signal mediation. TNF is produced by activated macrophages; however, its production by different cell types, such as astrocytes or endothelial cells, has also been demonstrated (McCall et al. 1989; Schall et al. 1990; Vilcek and Lee 1991). TNF was originally discovered in trials to treat cancer patients by infecting them with bacteria (Carswell et al. 1975). It induces large areas of necrosis in tumours, transplanted into nude mice. Histologically, it is well known that small cell lung carcinomas in particular often reveal large areas of infarct-like necrosis. Our data reveal a remarkable relationship of the presence of TNF-binding sites to the cell type: small cell lung carcinomas and epidermoid carcinomas exhibit detectable binding sites to TNF in about 60–70% of the tumours. This differs from the findings in large cell carcinomas and adenocarcinomas (18% and 45% respectively). The specific antibody applied against TNF showed a positive staining in more than 70% of the small cell lung carcinomas, and in only 12/32 (38%) cases of the non-small cell lung carcinomas. Only 3/10 epidermoid carcinomas revealed a presence of TNF within the tumour cells. The presence of binding sites to TNF is strongly associated with the presence of the TNF protein in the tumour cells (73% of the cells with detectable TNF-binding sites showed intracellular TNF); however, about 25% of the tumours with TNF-binding sites were apparently devoid of detectable intracellular TNF. In addition, 45% of the tumour cells without detectable TNF (Table 3). Thus, there is a close, however, not complete overlap between the presence of immunologically detectable intracellular TNF and the binding sites, visualized by biotinylated TNF.

A similar correlation of expression was observed between TNF-binding sites and the staining behaviour of NSE antibody. This association between TNF-binding sites and immunologically reactive NSE holds true for small cell lung carcinomas and non-small cell lung carcinomas. Although no association of the expression of

neuroendocrine features and the survival of patients was observed, there is evidence that small cell lung cancers with neuroendocrine features show a better response to therapeutic drug regimes followed by a pronounced drug resistance after tumour relapse, when compared with their non-neuroendocrine counterparts (Kayser et al. 1991). The close association of NSE-staining behaviour and TNF-binding sites may indicate that immunoresponse mechanisms may be involved to explain this observation.

It is also noteworthy that a close association of the expression of TNF-binding sites and lactose-specific endogenous lectins is apparent (Table 3). All non-small cell lung carcinomas, showing lactose-specific endogenous lectins, revealed TNF-binding capacity, detectable by our technique. Only the small cell lung carcinoma included a few tumours (6/29) which stained positively for biotinylated lactose-specific neoglycoprotein and negatively for biotinylated TNF. Endogenous lactose-specific lectin is usually not detectable at the probe concentration applied with this technique in normal lung parenchyma, and is seen less frequently in human lung carcinomas than maltose-, mannose- or fucose-specific sugar receptors (Kayser et al. 1989b).

A few cases were found that were devoid of binding sites to biotinylated ML-1. The staining behaviour of the intra-tumour mononuclear inflammatory cells was usually stronger than that of the normal lung parenchyma and of the tumour cells (Fig. 3). The data are in agreement with previous reports about the non-specific activation of immune variables like serum levels of cytokines by application of nanogram quantities of ML-1 (Gabius 1991; Gabius et al. 1991b).

From the technical point of view, histochemical application of biotinylated TNF is of similar value to that of epidermal growth factor (EGF). Kayser et al. (1990a, b) have demonstrated that biotinylated EGF is a useful histochemical probe for analysis of EGF-specific binding sites. In contrast to the application of EGF-receptor-specific monoclonal antibodies, biotinylated EGF detects all binding sites (including the EGF-specific receptors) independent of the mammalian species of the tissue. The same statement holds true for the application of biotinylated TNF, which is easy to perform on formalin-fixed, paraffin-embedded tissue. Our report extends the use of biotinylated TNF for receptor detection from native cells, as reported recently by Ranges et al. (1989), to fixed human lung material.

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