Expression of cadherin and NCAM in human small lung cancer cell lines and xenografts

K. Rygaard1, C. Møller2, E. Bock2 & M. Spang-Thomsen1

1University Institute of Pathological Anatomy, University of Copenhagen, Frederik V's Vej 11, DK-2100 Copenhagen Ø; 2Research Centre for Medical Biotechnology, The Protein Laboratory, University of Copenhagen, Sigurdsvej 34, DK-2200 Copenhagen N, Denmark.

Summary Tumour cell adhesion, detachment and aggregation seem to play an important part in tumour invasion and metastasis, and numerous cell adhesion molecules are expressed by tumour cells. Several families of cell-cell adhesion molecules have been described, of which two groups are particularly well characterised, the cadherin family and the Ig superfamily member, neural cell adhesion molecule (NCAM). We investigated expression of these two adhesion molecule families in small cell lung cancer (SCLC) cell lines and xenografts by immunoblotting. Nineteen tumours established from 15 patients with SCLC were examined. All tumours but one expressed both cadherin and NCAM. The tumours expressed one, two or rarely three cadherin bands, and different combinations of two major isoforms of NCAM with M's of approximately 190,000 and 135,000. Polysialylation of NCAM, a feature characteristic of NCAM during embryonic development, which may play a role in connection with tumour invasion and metastasis, was found in 14/18 NCAM expressing SCLC tumours. Individual tumours grown as cell lines and as nude mouse xenografts showed no qualitative differences in cadherin or NCAM expression.

Small cell lung cancer (SCLC) is a common human malignancy with an aggressive behaviour and a high tendency to metastasize. Tumour cell adhesion, detachment and aggregation seem to play an important part in tumour invasion and metastasis, and cell adhesion molecules are frequently expressed by tumour cells (Nicolson, 1988; Thiery et al., 1988; Linneimann et al., 1989; Shimoyama et al., 1989). Cell-cell adhesion molecules may be classified into several groups (Takeichi, 1991; Bock, 1991; Linneimann & Bock, 1989) of which the cadherin family constitutes one important group, and the Ig superfamily member, neural cell adhesion molecule (NCAM) another. Both these families of adhesion molecules have been attributed a role in embryonic development and both are expressed in certain forms of cancer as well as in several normal adult tissues (Thiery et al., 1988).

Cadherins are a family of cell adhesion molecules of which several subclasses are well characterised. Four major sub-classes are termed N-cadherin (M, 127–135,000), E-cadherin (M, 124,000) (also designated uvomorulin), P-cadherin (M, 118,000), and L-CAM (M, 124,000) (Takeichi, 1991). The cadherin family members are differentially expressed during morphogenesis, and may play a role in tumour invasion and metastasis, but the functional differences between the members have not been clarified.

At present, only little is known about cadherin expression in SCLC (Shimoyama et al., 1989).

NCAM is widely expressed during embryonic development as well as in adult neural and several other tissues and in certain tumours (Schol et al., 1988; Thiery et al., 1988). It exists in several different isoforms varying in their Mr, mainly due to differences in length of the C-terminal end of the molecule (for reviews, see Nybroe, Linneimann & Bock, 1988; Linneimann & Bock, 1989). All isoforms are encoded by the same gene due to alternative mRNA splicing (Owens, Edelman & Cunningham, 1987; Santoni et al., 1989). Three isoforms with Mr's of approximately 190, 135 and 115 kD are most commonly expressed, and are therefore termed major isoforms. The specific functional role played by each of these isoforms is not known.

A characteristic of NCAM is that it may contain various amounts of α2,8-linked polysialic acid (PSA) side chains (Finne, 1982), which may profoundly alter its homophilic binding abilities (Hoffman & Edelman, 1983; Rutishauser et al., 1988), and thereby possibly the invasive and metastatic capacity of the cells expressing NCAM.

NCAM expression has been reported to be present in almost all cases of SCLC (Souhami et al., 1991; Kibbelaar et al., 1989; Aletsee-Utrecht et al., 1990). Most previous studies of NCAM expression in SCLC have utilised immunohistochemical investigation. This method gives information about the subcellular localisation of NCAM, but it does not enable distinction between the different isoforms, neither does it provide detailed information about NCAM polysialylation, although demonstration of polysialylation is possible with appropriate antibodies (Kibbelaar et al., 1989; Souhami et al., 1991).

In the present study cell lines and nude mouse xenografts of SCLC tumours established from 15 patients were investigated for expression of cadherin and NCAM products by Western blotting. This method enables a semiquantitative determination of the level of adhesion molecule expression. It also allows determination of the Mr of the expressed cadherin and NCAM molecules, and an estimation of the degree of NCAM polysialylation. The use of tumour material grown in two different model systems provides information about whether expression of adhesion molecules may be influenced by the growth conditions of the tumour cells or whether it is an inherent characteristic of the cells.

The study is the first report of cadherin expression in a large panel of SCLC tumours based on immunoblotting, and it provides new data on the expression of NCAM isoforms in this disease. Furthermore, comparison of expression levels of adhesion molecules in cell lines and xenografts in nude mice has not been published previously.

Materials and methods
Cell lines and tumour xenografts

Nineteen tumours established from 15 patients were investigated. Four tumours were grown only as xenografts, one tumour only as cell line, while the remaining were investigated both as cell lines and as xenografts.

Cell lines were grown at 37°C in a humidified atmosphere containing 5% CO2. Media contained 10% foetal calf serum. Tumours designated CPH were established in Copenhagen, Denmark (Engelholm et al., 1986). DMS tumours at
Dartmouth Medical School, NH, USA (Pettengill et al., 1980), the NCI tumour at the National Cancer Institute, MD, USA (Carney et al., 1985), and GLC tumours at University Hospital of Groningen, The Netherlands (de Leij et al., 1985; Berendse et al., 1988). CPH-54A and CPH-54B are in vitro established subclones of the same original tumour (Engelholm et al., 1985). GLC-14, GLC-16, and GLC-19 were established from the same patient during longitudinal follow-up (Berendse et al., 1988). Cell lines were regularly tested and found free of Mycoplasma infection.

Cells for investigation were harvested in mid- to late exponential growth phase. Harvesting was done by scraping with a rubber policeman for cells growing attached to the bottom of culture flasks, and by aspirating cells growing as floating aggregates. The cells were washed in sterile buffer (150 mM NaCl; 10 mM EDTA; 10 mM Tris; pH 8.0), spun down, immediately frozen in liquid nitrogen, and stored at −80°C until further processing.

Xenografts were established in the flanks of nude mice by s.c. inoculation of 10⁷–10⁸ cells from cell lines, or directly from patients by inoculation of 2-mm-diameter tumour blocks (Spang-Thomsen, Norden & Visfeldt, 1980). Serial transplantation was performed by s.c. inoculation of 2-mm-diameter tumour blocks under general anesthesia. The mice were of NMRI or BALB/c origin and in specific pathogen-free status; they were kept in laminar-air flow clean benches. Sterile food and water were given ad libitum.

Tumour samples for investigation were cut free of visible necrotic tissue, immediately frozen in liquid nitrogen, and stored at −80°C.

Protein extraction, electrophoresis and immunoblotting

Cell and tissue samples for proteins extraction were homogenised in lysis buffer (25 mM Tris (pH 7.5); 50 mM NaCl; 0.5% (w/v) sodium-deoxycholate; 1% (v/v) Nonidet P-40; 0.1% (w/v) sodium dodecyl sulfate (SDS); 1 mM phenylmethylsulfonyl fluoride (PMSF); 500 KIE/ml aprotinin (Trasylol, Bayer), homogenised by ultrasonication, and centrifuged for 15 min at 12,000 g. The supernatant was transferred to a new tube and the protein concentration determined by the Bradford method (Bradford, 1976) using a commercial kit (Bio-Rad, CA). Sample buffer was added to the supernatant to give a final protein concentration of 2 μg μl⁻¹. The samples were boiled for 5 min and size-fractured on a preparative polyacrylamide gels (SDS-PAGE, Laemmli, 1970) on a ‘Phast’ SDS-PAGE electrophoresis unit (Pharmacia, Sweden). In cadherin experiments 2 μg total protein samples were electrophoresed per lane (eight lanes per gel), in NCAM experiments 8 μg (six lanes per gel), M₅, was determined with reference to molecular weight markers in the range from 44,000 to 200,000 (Bio-Rad, CA) and to NCAM bands in human brain and cadherin bands in newborn rat brain.

The electrophoretically separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, 0.22 μm, Millipore, France) by semi-dry electroblotting (Kyhse-Andersen, 1984) according to the manufacturers instructions (JKA, Copenhagen, Denmark). Membranes were blocked for 4 min in washing buffer (50 mM Tris, 350 mM NaCl; 0.1% (w/v) Tween-20 and 0.1% (w/v) BSA, pH 10.2) containing 2% Tween-20, washed for 3 × 10 min in washing buffer and subsequently incubated for 2 h with the relevant antibody.

The polyclonal cadherin antibody was raised in rabbits against a fusion protein produced from a combination of the β-galactosidase gene and a cDNA encoding amino acids 717–719 of chicken N-cadherin. The antibody reacts with N-cadherin in rat and human brain and with rat muscle, rat heart and rat liver (A.-M. Dalseg, K. Edvardsen & E. Bock, unpublished data). Comparison of the antibody with the N-cadherin antibody raised by Lagunovich et al. (1990), kindly supplied by the authors, has shown identical reactivity with all tested rat tissues. However, we cannot exclude the possibility that our N-cadherin antibody may also react with other cadherin family members, which are known to have pronounced homology in their C-terminal part. The reactivity of the cadherin antibody could be completely eliminated by pre-incubation with the fusion protein used for immunisation (data not shown).

For NCAM immunoblotting, a polyclonal rabbit antibody was used, which recognises all three major NCAM isoforms irrespective of the presence or absence of polysialylation (Moolenaar et al., 1990); the specificity of this antibody has been described previously (Rasmussen et al., 1983). Following incubation with primary antibody, the membranes were washed and incubated for 1 h with alkaline phosphatase-conjugated swine anti-rabbit antibody (Dukopatts, Glosstrup, Denmark) diluted 1:1000. After washing, bound antibody was visualised by a chromogenic reaction catalysed by the conjugated alkaline phosphatase using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the chromogenic substrate.

Results

The results are summarised in Table 1. Expression of both cadherin and NCAM was found in all investigated tumours except DMS-114 (Figures 1 and 2). The tumours expressed one or two cadherin bands (e.g. CPH-54B and DMS-92) of slightly different size, the M₅ of both being around 125–130,000. The larger band, which was expressed in all cadherin-positive tumours is most likely N-cadherin, since this band co-migrated closely with the N-cadherin band seen in rat brain. The smaller cadherin band recognised by the antibody may represent any of the other cadherin family members. NCAM was expressed as isoforms with M₅'s of approximately 190,000 and 135,000. Some tumours expressed mainly the 135,000 isoforms (e.g. CPH-186) whereas other tumours expressed both isoforms (e.g. DMS-406). In most of the tumours a cloudy area was visible on the Western blots above the bands caused by one of the major NCAM isoforms (e.g. DMS-92). This smear most likely represented polysialylation of NCAM, since varying degrees of polysialylation increases the molecular weight of NCAM to different extents, thus causing a smear on Western blots.

Extracts of human and rat brain, which were included on all blots as positive controls for NCAM and cadherin, respectively, were always positive.

There was a tendency towards higher levels of expression of both cadherin and NCAM in tumours propagated in vitro than in the same tumour grown in nude mice. However, there was no qualitative difference in the expression, since all tumours expressed the same cadherin and NCAM bands in the two model systems (Table 1).

Substitution of the primary antibodies with non-immune rabbit sera gave no bands which could be confused with the bands caused by adhesion molecules under study (data not shown). To exclude presence of soluble adhesion molecules in calf serum which was part of the cell growth medium, serum samples were electrophoresed and immunoblotted as described above. Neither cadherin, nor NCAM was detected in the calf serum.

Discussion

Eighteen of the 19 investigated SCLC tumours expressed both N-cadherin and one or more NCAM isoforms. This high frequency of expression of cell-cell adhesion molecules in SCLC indicates that adhesion molecules have important functions in this disease.

We found expression of N-cadherin in 18/19 SCLC tumours and thus demonstrate that cadherin expression in SCLC is very common. Using immunohistochemistry, E-cadherin and P-cadherin expression has previously been demonstrated in 44 lung carcinomas (Shimoyama et al., 1989), of which 2/2 SCLC cell lines were found to be positive.

In ten of the 18 cadherin expressing tumours the Western
Figure 1  Representative Western blots demonstrating cadherin expression in SCLC cell lines and xenografts. The position of the cadherin band(s) is indicated by arrows.

Figure 2  Representative Western blots demonstrating NCAM expression in SCLC cell lines and xenografts. The position of the 190.000 and 135.000 M, isoforms is indicated by arrows. Polysialylation is seen in some of the tumours (e.g. DMS-92), see Table I and text.

Table I  Expression of cadherin and NCAM proteins in SCLC cell lines and xenografts

| Tumour     | Cadherin Line | Cadherin Xeno | NCAM Line | NCAM Xeno |
|------------|---------------|---------------|-----------|-----------|
| CPH-54A    | + + + [S]     | + [S]         | + [A,B,P] | + [A,B,P] |
| CPH-54B    | + + + [S]     | + [S]         | + [A,B,P] | + [A,B,P] |
| CPH-136A   | NA + + [D]    | NA [A,B]     | + + [B]   | + [B]     |
| CPH-136B   | NA + + [D]    | NA [A,B]     | + + [B]   | + [B]     |
| CPH-167    | NA + + [D]    | NA [A,B]     | + + [B]   | + [B]     |
| CPH-186    | NA + + [D]    | NA [A,B]     | + + [B]   | + [B]     |
| DMS-33     | + + + [S]     | + [S]         | + [A,B,P] | + [A,B,P] |
| DMS-79     | + + + [S]     | + [S]         | + [A,B,P] | + [A,B,P] |
| DMS-92     | ++ + [D]      | ++ [D]        | + + [A,B,P] | + + [A,B,P] |
| DMS-114    | -             | -             | -          | -          |
| DMS-153    | ++ + [D]      | ++ [D]        | + + [A,B,P] | + + [A,B,P] |
| DMS-273    | (+)           | + [S]         | + [A,B]   | (+)       |
| DMS-406    | ++ + [S]      | NA ++ [A,B,P] | NA [A,B] | + [A,B]   |
| DMS-450    | ++ + [D]      | ++ [D]        | + [A,B,P] | + [A,B]   |
| GLC-13     | ++ + [D]      | ++ [D]        | + [A,B,P] | + [A,B]   |
| GLC-14     | + + + [D]     | + [D]         | + [A,B,P] | + [A,B]   |
| GLC-16     | + + + [D]     | + [D]         | + [A,B]   | + [A,B]   |
| GLC-19     | + + + [D]     | + [D]         | + [A,B]   | + [A,B]   |
| NCI-H69    | + [S]         | + [S]         | + + [A,B,P] | + + [A,B,P] |

The level of expression was rated visually as none: '-', trace: '(+)’, weak: '+', moderate: '+ +' or high: '+ + +'. For cadherin the presence of a single '[S]' or double/triple '[D]' band on Western blots is indicated. Expression of the major NCAM isoforms NCAM-190 and NCAM-135 is indicated as '[A]' and '[B]', respectively. Polysialylation of NCAM is indicated by '[P]'. NA, not available.
blots showed more than one band (Table I, Figure 1). Cadherin antibodies binding to the C-terminal part of cadherin, as does the antibody used in this study, has been reported to recognise both E-, N-, and P-cadherin (Geiger et al., 1990). N-cadherin is the largest of the known cadherins, and the larger band found in all cadherin expressing tumours is very likely to be N-cadherin. This assumption is favoured by the fact that the large band always co-migrated closely with the N-cadherin band in rat brain tissue. The smaller band found in ten of the tumours may represent one of the other described cadherin family members, a breakdown product of N-cadherin, or yet unidentified members of the cadherin family. With the increasing knowledge about the complexity of the cadherin family, it is evident that some caution must be exercised when interpreting results obtained with antibodies with broad cadherin family reactivity. Four cadherin subclasses have been well characterised, namely E-, N-, P-cadherin and L-CAM (Takeichi, 1991) but several more may exist (Suzuki, Sano & Tamhara, 1991).

NCAM was expressed in 18/19 of the tumours. This is in agreement with previous reports of very frequent expression of NCAM in SCLC (Schol et al., 1988; Kibbelaar et al., 1989; Aletsee-Ufrecht et al., 1990). While NCAM has for some time been known to be expressed in SCLC, little was known about which isoforms of NCAM that were expressed in this disease (Souhami et al., 1991). This is due to the fact that the majority of previous studies have employed immunohistochemical methods for demonstrating NCAM expression such as allow distinction between the various NCAM isoforms. In one study of NCAM expression in seven SCLC cell lines by immuno-blotting (Aletsee-Ufrecht et al., 1990), only the NCAM-135 isoform was demonstrated (termed NCAM-140 in their study). In two other previous studies in which SCLC NC-1H69 was included (Kibbelaar et al., 1989; Moolenaar et al., 1990), this tumour was shown to express both NCAM-190 and NCAM-135. The expression of these two major isoforms in NC-1H69 was confirmed in the present study. We found coexpression of NCAM-190 and NCAM-135 in more than half of the examined tumours, demonstrating that, in SCLC, two NCAM isoforms are commonly co-expressed. Since the role of the different NCAM isoforms is not settled, the possible biological implications of the difference in NCAM isoform expression between various SCLC tumours remains obscure.

It has recently been demonstrated, that the antigen recognised by SCLC cluster one (Cl1) antibodies (Souhami et al., 1991) is identical to NCAM (Patel et al., 1989; Moolenaar et al., 1990). Most SCLC tumours investigated so far have been found to express NCAM, while expression is found in only a few non-SCLC lung tumours (Mooi et al., 1988; Kibbelaar et al., 1989; Aletsee-Ufrecht et al., 1990).

An example of polysialylation in 14/18 tumours (Table I), but we cannot rule out the possibility that a lower level of polysialylation may be present in the tumours scored as negative. Low levels of polysialylation may be detectable with antibodies with high affinity for e2,8-linked polysialic acid side chains. Polysialylation of NCAM profoundly reduces the homophilic binding properties of cells (Rutishauser et al., 1988) and may facilitate migration of the cells due to altered cell-cell interactions. Thus, it could be speculated that the presence of polysialylation of NCAM on SCLC cells may contribute to the highly metastatic behaviour of this tumour type.

Qualitatively, the expression pattern of cadherin and NCAM was independent of whether the individual tumours were grown in vitro as cell lines or in vivo as nude mouse xenografts. However, a tendency towards higher expression in cell lines than in xenografts was noted. The difference may to some extent be caused by the presence in xenografts of murine stroma, which causes a dilution of the tumour cells. Despite the possible slight difference in the level of cadherin and NCAM expression between cell lines and xenografts, the demonstration of these adhesion molecules in both cell lines and in xenografts is evidence that expression is an inherent characteristics of the SCLC tumour cells, and not a phenotypic feature induced by the growth conditions. This notion is supported by the consistent demonstration of NCAM expression in SCLC in surgical biopsies (Mooi et al., 1988; Kibbelaar et al., 1989; Tome et al., 1990), as well as in cell lines established from patients with this disease (Kibbelaar et al., 1989; Moolenaar et al., 1990; Aletsee-Ufrecht et al., 1990).

It has been suggested that NCAM expression may be a feature specific of SCLC cell lines growing as floating aggregates in in vitro culture whereas cells growing adherent to the bottom of culture flasks express less or no NCAM (Doye et al., 1990). Our data do not support this hypothesis since the CPH cell lines, the DMS cell lines except DMS-79, and GLC-3 grew attached to the bottom of culture flasks. Thus, 10/14 cell lines expressed NCAM and grew attached.

At least three different types of cell-cell adhesion molecules are expressed on SCLC cells. Apart from cadherin and NCAM, also P integrin is expressed in a large proportion of SCLC cells (Feldman et al., 1991). Cell-cell adhesion molecules are likely to be involved in the process of metastasis (Nicolson, 1988; Thiery et al., 1988; Takeichi, 1991). However, it is not clear what influence adhesion molecules on the surface of cancer cells have on their ability to metastasise. On one hand adhesion molecules may retard the escape of tumour cells from the primary site due to increased adhesion to other cells and to intercellular matrix proteins, but on the other hand adhesion molecules may be necessary for the cells to attach to a secondary, i.e. metastatic, site. However, decreased metastatic potential of cells expressing NCAM was reported (Andersson et al., 1991) and also increased invasive and metastatic potential upon loss of cadherin expression (Behrens et al., 1989; Hashimoto et al., 1989; Vleminkx et al., 1991) has been described. Hence, the functional role of adhesion molecules in invasion and metastasis is complex, and it may seem surprising that SCLC which is highly metastatic in patients, expresses several types of adhesion molecules.

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