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Poly(α2,8-deaminoneuraminic acid) is expressed in lung on a single 150-kDa glycoprotein and is an oncodevelopmental antigen

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ABSTRACT **Homopolymers of α2,8-linked *N*-acetylneuraminic acid [poly(α2,8-Neu5Ac)] of the neural cell adhesion molecule NCAM have been shown to be temporally expressed during lung development and represent a marker for small cell lung carcinoma. We report the presence of a further polysialic acid in lung that consists of oligo/polymers of α2,8-linked deaminoneuraminic acid residues [poly(α2,8-KDN)], as detected with a monoclonal antibody in conjunction with a specific sialidase. Although the various cell types forming the bronchi, alveolar septs, and blood vessels were positive for poly(α2,8-KDN) by immunohistochemistry, this polysialic acid was found on a single 150-kDa glycoprotein by immunoblot analysis. The poly(α2,8-KDN)-bearing glycoprotein was not related to an NCAM protein based on immunochemical criteria. The expression of the poly(α2,8-KDN) was developmentally regulated as evidenced by its gradual disappearance in the rat lung parenchyma commencing 1 week after birth. In adult lung the blood vessel endothelia and the smooth muscle fibers of both blood vessels and bronchi were positive but not the bronchial and alveolar epithelium. The poly(α2,8-KDN)-bearing 150-kDa glycoprotein became reexpressed in various histological types of lung carcinomas and cell lines derived from them and represents a new oncodevelopmental antigen in lung.**

In vertebrates homopolymers of α2,8-linked *N*-acetylneuraminic acid [poly(α2,8-Neu5Ac)] represent a posttranslational modification found on the neural cell adhesion molecule NCAM (1, 2) and the α subunit of the voltage-gated sodium channels in brain (3). The poly(α2,8-Neu5Ac) of NCAM modulates the adhesive properties of NCAM and influences other cell adhesion molecules (4–8). Thereby, it plays a role in the development and differentiation of the nervous system (8–11) and probably of other organs as indicated by its spatiotemporal expression patterns in fetuses (12). In adults, poly(α2,8-Neu5Ac) becomes restricted to the nervous system and seems to be important in spatial learning and memory formation (13, 14). Transient reexpression of poly(α2,8-Neu5Ac) in adult tissues can occur during regeneration and repair after tissue damage (15, 16). In a variety of malignant human tumors, poly(α2,8-Neu5Ac) on NCAM represents an oncodevelopmental antigen (17–19).

During the formation of the respiratory tract, its endodermally derived epithelial elements temporally express poly(α2,8-Neu5Ac) on NCAM (12). In adult lung, however, only the disseminated neuroendocrine cells and nerve fibers were positive for poly(α2,8-Neu5Ac), and of the various types of lung carcinomas, only the highly malignant small cell lung carcinoma were positive (18, 19). Expression of poly(α2,8-Neu5Ac) in clonal sublines of small cell lung carcinoma was found to be correlated with their invasive and metastatic growth (20).

A polysialic acid different from poly(α2,8-Neu5Ac) that consists of homopolymers of α2,8-linked deaminoneuraminic acid [2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid or poly(α2,8-KDN)] was discovered in the reproductive organs of fish (21). The fish KDN-glycoproteins are polydisperse with regard to molecular mass ranging from 700 to 4000 kDa, and the KDN moieties may constitute up to 80% of their total molecular mass (22, 23). Recently, a monoclonal antibody (mAb) recognizing the poly(α2,8-KDN) glycan (24), and a bacterial sialidase (KDNase) specifically hydrolyzing KDN ketosidic linkages (25) were reported. These two reagents permitted, to our knowledge, for the first time the detection of poly(α2,8-KDN) in various organs of mammals, thereby proving that diversity in polysialic acids exists in higher vertebrates (24, 26). We report herein that poly(α2,8-KDN) exists in lung and, interestingly, is found on a single 150-kDa glycoprotein. Although poly(α2,8-KDN) was detectable in the lung of rat and human embryos and in rats up to 2 weeks old, it was undetectable in the alveolar and bronchial epithelium 3 weeks after birth and in adult rat and human. In various types of human malignant lung tumors, however, it was reexpressed and represented a new oncodevelopmental antigen.

MATERIALS AND METHODS

Reagents, Tissues, and Cells. For the detection of poly(α2,8-KDN), the mouse mAb kdn8kdn was used (24), which binds to this glycan exhibiting a degree of polymerization = 2–6 (27). Affinity-purified bacterial KDNase was prepared from *Sphingobacterium multivorum* as described (25). KDN-glycoprotein was purified from the vitelline envelope and the ovarian fluid of rainbow trout (28). Rabbit anti-human and anti-rat NCAM antibodies reactive with all major NCAM isoforms were kindly provided by E. Bock (University of Copenhagen, Denmark). mAb 735 directed against poly(α2,8-Neu5Ac) was kindly provided by D. Bitter-Suermann (Medical High School, Hannover, Germany) (29). Bacteriophage K1F endosialidase N (endo N), hydrolyzing poly(α2,8-Neu5Ac), was purified according to Petter and Vimr (30–32). The bacteriophage and the *Escherichia coli* EV1 (31) were a generous gift from E. Vimr (University of Illinois, Urbana). Alkaline phosphatase-conjugated and biotinylated affinity-purified goat anti-mouse IgG+IgM antibodies, gold-labeled (4 nm and 8 nm) affinity-purified rabbit anti-horseradish peroxidase antibodies and streptavidin-horseradish peroxidase conjugates were purchased from Jackson ImmunoResearch; glutaraldehyde (vacuum distilled), paraformaldehyde, hydroquinone, and silver acetate were from Fluka; 5-bromo-4-chloro-3-indolyl phos-

Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; KDN, 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid or naturally occurring deaminoneuraminic acid; KDNase, an enzyme that catalyzes the hydrolysis of ketosidic linkages of KDN; endo N, a bacteriophage K1F-derived endosialidase hydrolyzing poly(α2,8-Neu5Ac); mAb, monoclonal antibody.

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phate (BCIP) and nitroblue tetrazolium (NBT) were from Sigma; and immunobeads from PerSeptive Diagnostics (Cambridge, MA).

Rat lung (embryonic day 20, postnatal days 2, 7, 14, 21, and 28, and adult) was fixed either by immersion or vascular perfusion through the right heart with 3% formaldehyde/0.1% glutaraldehyde in Hanks' balanced salt solution for a total of 4 h and embedded in paraffin. Formaldehyde-fixed and paraffin-embedded human embryonic and adult lung and lung carcinomas (five squamous cell carcinomas, five adenocarcinomas, and five small cell lung carcinomas) were obtained from our archival files.

Human small cell lung carcinoma cell line NCI H69 (American Type Culture Collection) and lung squamous cell carcinoma lines Calu and Hotz as well as lung adenocarcinoma cell lines A125 and A549 (kindly provided by R. Stahel, Clinical Oncology, University Hospital Zürich) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a humid atmosphere containing 5% CO_2.

Immunohistochemistry. For immunolabeling, deparaffinized and rehydrated tissue sections were conditioned with PBS (10 mM sodium phosphate buffer, pH 7.2/0.15 M NaCl) containing 1% bovine serum albumin (BSA), 0.05% Triton X-100, and 0.05% Tween 20 for 10 min at room temperature followed by incubation with the mAb kdn8kdn (1.5–2.5 μg/ml) for 18 h at 4°C. After adaptation to room temperature, sections were processed by a streptavidin/biotin immunogold-silver technique (33) and counterstained with nuclear fast red.

In controls, sections were pretreated with KDNase (8 units/ml; for definition of unit, see ref. 25) in 100 mM Tris-acetate buffer (pH 6.0) for 24 h at 25°C, in the Tris-acetate buffer not containing KDNase or incubated with antigen-preabsorbed mAb kdn8kdn (5 μg of KDN glycoprotein in 25 μl of PBS mixed with 50 μl of mAb kdn8kdn at 2.5 μg/ml). In other controls the mAb kdn8kdn was omitted.

Analytical Procedures. Pieces from embryonic, postnatal, and adult rat lung tissue and the various human lung carcinoma cell lines were homogenized at 4°C in PBS (pH 7.4) containing 1% Triton X-114, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin. After centrifugation (10,000 × *g*, 5 min), proteins of the supernatant were kept in Laemmli buffer (34) at 65°C for 15 min (17), followed by SDS/PAGE in 3–10% polyacrylamide gradient gels. Immunoblot analysis on nitrocellulose or poly(vinylidene difluoride) (PVDF) membranes was performed according to Towbin *et al.* (35) but using a semi-dry blotting apparatus. Briefly, strips were blocked with PBS containing 1% [for detection of poly(α2,8-KDN)] or 2% [for detection of poly(α2,8-Neu5Ac) and NCAM] defatted milk powder and 0.05% Tween 20 for 1 h at room temperature, incubated with mAb kdn8kdn (1.3 μg/ml) or mAb 735 (5 μg/ml) for 18 h at 4°C or anti-NCAM antibodies for 1 h at ambient temperature followed by alkaline phosphatase-labeled goat anti-mouse IgG+IgM antibodies or goat anti-rabbit IgG and a nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color reaction.

In controls, strips were incubated with KDNase as described above, antigen-preabsorbed mAb kdn8kdn, and endo N for 60 min at 37°C.

For immunoprecipitation, goat anti-rabbit IgG immunobeads were incubated first with PBS containing 1% BSA and 0.05% of Triton X-100 and Tween 20 for 1 h at 4°C followed by incubation with anti-NCAM antibodies for 18 h at 4°C and two washes with PBS. Homogenate of postnatal day 2 rat lung was centrifuged at 10,000 × *g* for 15 min at 4°C, and the supernatant (1 mg of protein per 1 ml of PBS containing 1% BSA and 0.05% Tween 20) was incubated with the anti-NCAM immunobeads overnight at 4°C. After two washes with PBS, the pelleted immunobeads (endo N treated or not) were incubated in 1 ml of 60 mM Tris·HCl (pH 6.8) containing 2% SDS and 5% 2-mercaptoethanol at 65°C for 15 min. The supernatant was then examined by immunoblot analysis for immunoreactivity of poly(α2,8-KDN), poly(α2,8-Neu5Ac), and NCAM as described above.

RESULTS

Immunoblot analysis of rat lung with mAb kdn8kdn revealed the presence of an immunoreactive band of 150 kDa at embryonic day 20 that was detectable with the same intensity until postnatal day 7 (Fig. 1*A*). Thereafter, the intensity of the immunoreactivity at 150 kDa decreased to become basically undetectable in adult lung (Fig. 1*A*). In controls, when the strips were pretreated with KDNase (Fig. 2, lane 3) or when antigen-preabsorbed mAb kdn8kdn was applied (Fig. 2, lane 4), no immunoreactive band was detectable in postnatal day 2 lung. Immunoblot analysis of the pellet obtained from different rat organs after Triton X-114 extraction showed only trace amounts of immunoreactivity. A comparison with mAb 735 to detect poly(α2,8-Neu5Ac) (29) in rat lung is shown in Fig. 1*B*. A broad band of ≥200 kDa was detectable (see also ref. 12) that increased in intensity from embryonic day 20 to postnatal day 14 to become weaker thereafter and to form only a faint band in adult lung that is due to immunoreactive nerve fibers and dispersed neuroendocrine cells (12). The distinct band seen for poly(α2,8-KDN) compared with the broad one for poly(α2,8-Neu5Ac) may be due to a lower degree of polymerization of poly(α2,8-KDN), a more uniform degree of polymerization compared with poly(α2,8-Neu5Ac), and the different epitope size requirements for mAb kdn8kdn compared with mAb 735 (27). A complex pattern of NCAM protein immunoreactive bands was observed in embryonic and postnatal lung samples not treated with endo N (data not shown), which was apparently due to the differential degree of poly-

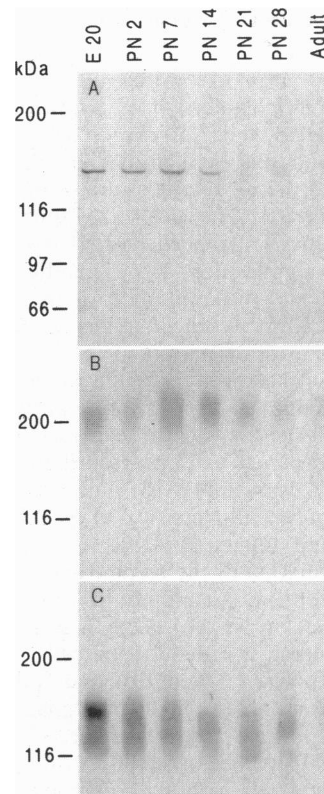

FIG. 1. Immunoblot analysis for poly(α2,8-KDN) (*A*), poly(α2,8-Neu5Ac) (*B*), and NCAM proteins (*C*) of rat lung of embryonic day 20 (E 20), various postnatal (PN) time points from day 2 until day 28, and adult animals. The protein samples used in *C* were pretreated with endo N to remove poly(α2,8-Neu5Ac).

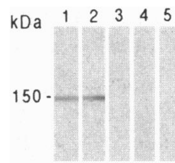

FIG. 2. Immunoreactivity in postnatal day 2 rat lung is specific for poly(α2,8-KDN). Positive control (lane 1) and control with KDNase buffer (lane 2). The immunoreactive band becomes undetectable after KDNase pretreatment (lane 3), antigen-preabsorption of the mAb (lane 4), and omittance of the mAb (lane 5).

sialylation. Immunoblot analysis of endo N-pretreated samples of lung proteins revealed the presence of 140-kDa and 120-kDa NCAM isoforms (see also ref. 12) that with increasing age of the rats became weaker to result in adult rat lung in a faint 140-kDa band (Fig. 1*C*). To test whether poly(α2,8-KDN) immunoreactivity was present on immunoprecipitated and endo N-treated NCAM of postnatal day 2 rat lung, immunoblot analysis with mAb kdn8kdn was performed. A negative result was obtained, demonstrating that the 140- and 120-kDa NCAM isoforms do not carry poly(α2,8-KDN) (Fig. 3, lane 2). Regardless, endo N pretreatment of lung proteins to remove poly(α2,8-Neu5Ac) did not effect the detectability of the poly(α2,8-KDN) reactive band (data not shown).

Immunoblot analysis of poly(α2,8-KDN) in lung squamous cell carcinoma and adenocarcinoma lines and the H69 small cell lung carcinoma cell line revealed immunoreactivity associated with a single 150-kDa band (Fig. 4).

The incubation of paraffin sections of embryonic rat (Fig. 5*a*) and human and rat postnatal (Fig. 5*b*) lung with the mAb kdn8kdn resulted in intense cellular staining. In embryonic and postnatal rat lung, immunostaining for poly(α2,8-KDN) was found in the cells forming the alveolar septs and in the epithelium, smooth muscle fibers, and fibroblasts of the bronchi, and smooth muscle fibers of blood vessels. The goblet cell mucus was unreactive. Immunostaining was present along the cell surface and in a punctate pattern in the cytoplasm probably representing endosomal and lysosomal elements. Similar to what was observed by immunoblot analysis, the intensity of the immunostaining decreased in the lung of the postnatal rats and became undetectable in adult rat and human lung respiratory epithelium (Fig. 5*c*). Only the blood vessel endothelia and the smooth muscle fibers of blood vessels and bronchi remained positive. However, immunostaining for poly(α2,8-KDN) was again detectable in paraffin sections of squamous cell and adenocarcinomas and small cell lung carcinomas (Fig. 6) with the surrounding normal lung tissue being unreactive for poly(α2,8-KDN).

DISCUSSION

The findings that have emerged from the present study can be summarized in that a new type of polysialic acid, poly(α2,8-KDN), has been detected in mammalian lung where it represents an oncodevelopmental antigen. Thus, in addition to the poly(α2,8-Neu5Ac) on the neural cell adhesion molecule NCAM (12), poly(α2,8-KDN) exists in lung on a glycoprotein distinct from the NCAM proteins. It is remarkable that this newly discovered polysialic acid in lung appears to be present on a single 150-kDa glycoprotein. To our knowledge, this represents the first example of such highly selective protein expression of a carbohydrate structure. Currently, it is not known whether glycolipids in mammalian tissues carry poly(α2,8-KDN) as demonstrated for fish (36). Aside from tissue- and cell-type-specific expression patterns, various sialylated oligosaccharide side chains occur commonly on many different glycoproteins (37, 38). A somewhat more restricted expression on glycoproteins has been reported for 9-*O*-acetylated sialic acids (39), and for the poly(α2,8-Neu5Ac), which is found on the various isoforms of NCAM (1, 2), the sodium channel α subunit (3), and some yet unidentified tumor cell glycoproteins that are neither related to NCAM nor to sodium channels (40).

Both forms of polysialic acid in lung have in common a developmentally regulated expression in that they are present during certain stages of organogenesis and of postnatal life and are undetectable in the bronchial and alveolar epithelium of adult lung. However, the fine details of their spatiotemporal expression pattern are remarkably different. At the cellular level, for example, the poly(α2,8-KDN) was always coexpressed in epithelial and mesenchymal elements. In contrast, for poly(α2,8-Neu5Ac) such coexpression was observed only over a limited period of time in the embryo (chicken, rat, and human) after which it became restricted to the mesenchyme

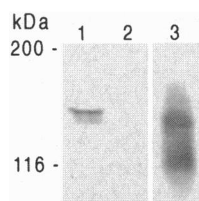

FIG. 3. The glycoprotein bearing the poly(α2,8-KDN) in postnatal day 2 rat lung is immunochemically not related to NCAM proteins. Lane: 1, immunoblot for poly(α2,8-KDN) of lung homogenate; 2, immunoblot for poly(α2,8-KDN) of immunoprecipitated lung NCAM; 3, aliquot of the NCAM immunoprecipitate used in lane 2 but probed with anti-NCAM antibodies.

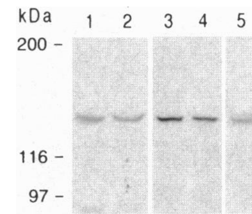

FIG. 4. Poly (α2,8 KDN) is detectable in human lung carcinoma cell lines. Lanes: 1 and 2, adenocarcinoma cell lines; 3 and 4, squamous cell carcinoma cell lines; 5, small cell lung carcinoma cell line H69.

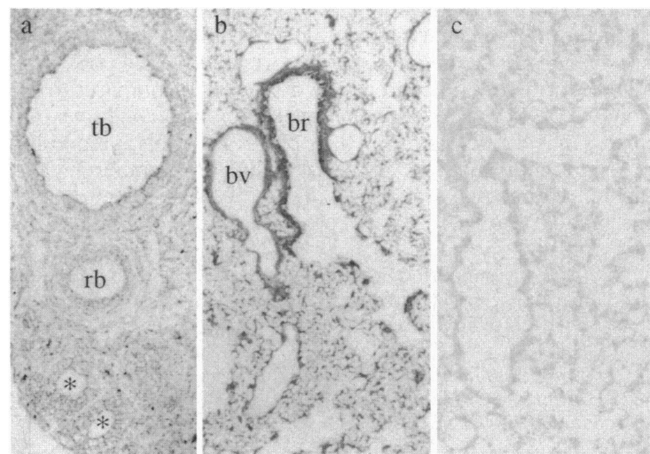

FIG. 5. Immunohistochemical detection of poly(α2,8-KDN) in paraffin sections of rat lung by immunogold-silver staining. The immunolabeling appears in black. (*a*) Immunostaining is observed in a terminal bronchiole (tb), a respiratory bronchiole (rb), and terminal sacs (asterisks) in embryonic day 18 rat lung. (*b*) Immunostaining in postnatal day 2 rat lung is present in the wall of a peripheral bronchus (br), which extends in a bronchiole, and in the alveolocapillary membranes and a blood vessel (bv). (*c*) Immunostaining is undetectable in the epithelium of bronchi and alveoli of adult rat lung.

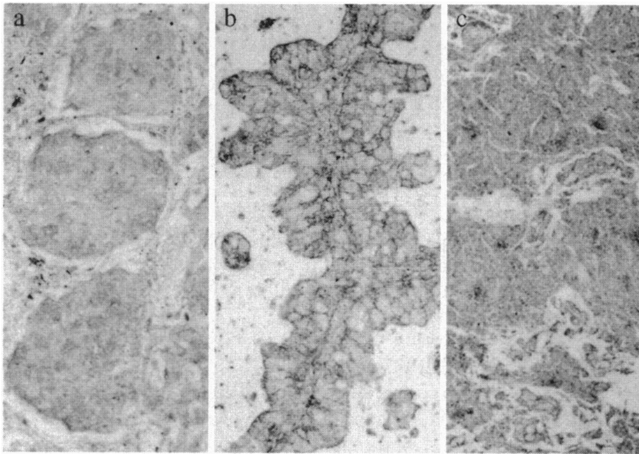

FIG. 6. Immunohistochemical detection of poly(α2,8-KDN) in paraffin sections of a human lung squamous cell carcinoma (*a*), an adenocarcinoma (*b*), and a small cell lung carcinoma (*c*).

and neuroendocrine cells (12). As already observed for the poly(α2,8-Neu5Ac) on NCAM (12, 18, 19), immunoreactivity for poly(α2,8-KDN) became undetectable in adult lung by Western blot analysis. The results of the *in situ* immunohistochemical investigations confirmed the absence of poly(α2,8-KDN) in the alveolar and bronchial epithelium. However, endothelial cells and smooth muscle fibers exhibited immunostaining as observed in various other adult rat tissues (26). Both types of polysialic acids have in common that they become reexpressed in malignant human lung tumors. The poly(α2,8-KDN)-bearing 150-kDa glycoprotein represents a valuable oncodevelopmental antigen being detectable in all different histological types of lung carcinomas. In contrast, the expression of poly(α2,8-Neu5Ac) of NCAM is selective and limited to the small cell lung carcinoma (18, 19, 41).

In normal lung and in lung carcinomas, the nature and possible function(s) of the 150-kDa glycoprotein as well as that of its poly(α2,8-KDN) moiety are unknown at present. It remains to be determined whether the poly(α2,8-KDN) in lung plays a role during organogenesis as was shown for poly(α2,8-Neu5Ac) on NCAM in other organs (10) and what it may contribute to the growth behavior of malignant cells (20). Further, we do not know whether the poly(α2,8-KDN) moiety or the entire molecule is absent in adult lung or whether there are changes in the degree of polymerization of the poly(α2,8-KDN) during organogenesis and, if so, how it compares to the poly(α2,8-KDN) detectable in lung carcinomas. *In vitro* experiments have demonstrated that poly(α2,8-KDN) and other polysialic acids bind Ca^{2+} preferentially (42) and thereby may influence unknown function(s) of the 150-kDa glycoprotein in the lung *in vivo*.

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