Histochem Cell Biol (2009) 131:383–390
DOI 10.1007/s00418-008-0533-5

ORIGINAL PAPER

Increased expression of glycodelin mRNA and protein in rat lungs during ovalbumin-induced allergic airway inflammation

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Accepted: 21 October 2008 / Published online: 12 November 2008
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Abstract Asthma is a chronic inflammatory disease accompanied by airway obstruction and hyper-responsiveness. Asthmatic inflammation is characterized by the expression of multiple genes for inflammatory mediators. Glycodelin is a glycoprotein with several functions in cell recognition and differentiation. There is substantial evidence that glycodelin may be a mediator for immunomodulatory and immunosuppressive effects on several human tissues. To determine the potential role of glycodelin in the pulmonary immune response, we examined the distribution of the glycodelin mRNA and protein in an experimental rat model of allergen-induced airway inflammation. The experimental model developed an airway response to inhaled nebulized ovalbumin in adult rats. Two groups of rats (ovalbumin and saline) were challenged for 3 weeks, lungs were fixed and embedded, and sections were studied for expression of glycodelin mRNA by in situ hybridization and protein by immunohistochemistry. Glycodelin is expressed in Clara cells of bronchial epithelium, type II pneumocytes and alveolar macrophages. Densitometric analyses show a significant increase of the glycodelin mRNA and protein expression in rat lungs after ovalbumin challenge. Induced glycodelin amounts in tissue, particularly in Clara cells and alveolar macrophages were found. The altered expression pattern of glycodelin may contribute to the pulmonary immune response in asthmatic inflammation.

Keywords Glycodelin · Rat · Lung · Allergic airway inflammation

Introduction

Asthma is a chronic inflammation disease of the lung accompanied by reversible airflow obstruction, airway hyper-responsiveness and airway inflammation (Bousquet et al. 2000). Asthmatic inflammation is characterized by the expression of multiple genes for inflammatory mediators such as cytokines, leucotrienes, adhesion molecules, enzymes and receptors. Infiltration of eosinophils and T lymphocytes, goblet-cell hyperplasia, smooth-muscle-cell hyperplasia and neovascularization predominate in chronic asthma (Djukanovic et al. 1990; Beasley et al. 1993; Rogers 2002). The disease is characterized by a break of immunological tolerance and in both Th1- and Th2-driven mechanisms may be involved. Moreover, patients suffering from severe chronic atopic diseases have been shown to develop autoreactive antibodies and vice versa autoreactive IgE antibodies may contribute to the perpetuation of allergic immune responses. Garn et al. (2007) demonstrate that allergic sensitization and subsequent airway aerosol challenge of mice to a potential auto-antigen leads to an allergic airway inflammation and chronic exposure of sensitized

Results of this study were presented in part on the 49th Symposium of the Society of Histochemistry 2007 in Freiburg

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mice to a foreign allergen induces autoreactive antibodies. Recently it was shown that female mice are more susceptible to the development of allergic airway inflammation than male mice (Melgert et al. 2005). It was speculated that high levels of oestrogen receptor activation via endogenous oestrogen could be pro-asthmatic. There is further evidence for an association of asthma and intake of contraceptive in humans and a risk for asthma exacerbations during pregnancy (Jensen-Jarolim and Untersmayr 2008).

Glycodelin (Gd), previously known as placental protein 14 (PP14; Bohn et al. 1982), and progesterone-associated endometrial protein (PAEP; Kämäräinen et al. 1991), is a homodimeric 28 kDa glycoprotein and a member of the immunocalins, a lipocalin subfamily (Logdberg and Wester 2000). Glycodelin synthesis and secretion is spatially and temporally regulated. A significant positive correlation has been found between serum estradiol, progesterone, relaxin as well as human chorionic gonadotropin concentration and endometrial Gd staining (Waites and Bell 1989; Stewart et al. 1997; Taylor et al. 1998; Tseng et al. 1999; Toth et al. 2008).

In the human reproductive tract, Gd is differentially glycosylated in a tissue-specific manner (Dell et al. 1995; Morris et al. 1996). Depending on its carbohydrate structure there is substantial evidence that Gd may be a mediator for immunomodulatory and immunosuppressive effects on several human tissues. Glycodelin A (GdA) suppresses the release of interleukin-2 and interleukin-2 receptor from stimulated lymphocytes (Pockley and Bolton 1989, 1990). It also inhibits the cytosolic capacity of natural killer cells (Okamoto et al. 1991). Gd is also a glycoprotein with contraceptive properties, since it inhibits binding of human sperm to the zona pellucida (Oehninger et al. 1995). On the other hand Gd is a marker of epithelial differentiation (Arnold et al. 2001, 2002).

Under physiological conditions, Gd is mainly localized in organs of the female and male reproductive tract from rats, baboons and humans (Joshi et al. 1981; Mazurkiewicz et al. 1981; Fazleabas et al. 1997; Keil et al. 1999). Gd can be synthesized by the endometrial tissue and the decidua during pregnancy (Julkunen et al. 1985, 1986; Julkunen 1986; Kämäräinen et al. 1998). Otherwise, both Gd and its mRNA have also been found in glandular tissues, e.g. in parabronchial and eccrine sweat glands (Kämäräinen et al. 1997) as well as erythroid precursors of human bone marrow cells (Kämäräinen et al. 1994).

The abnormal gene expression of Gd plays an important role in the pathogenesis of different diseases such as endometrial, ovarian, breast and cervical carcinoma, first trimester abortion and mole pregnancy as well as intrauterine growth restriction and haemolysis elevated liver low platelet (HELLP) syndrome (Kämäräinen et al. 1996; Horowitz et al. 2001; Shabani et al. 2005; Jeschke et al. 2005a, b; Richter et al. 2007; Toth et al. 2008).

Nevertheless, these findings on Gd localization, regulation and expression in different diseases demonstrate that glycodelin can not be viewed as a pregnancy-specific protein to modulate potentially harmful immune responses. Altogether, the complex immunomodulatory properties of Gd, the association of asthma with irregular menstruation and pregnancy as well as with intake of hormones, stimulated us to investigate a possible localization of Gd in inflammatory and other cells of the lung in an experimental model of allergic disease.

Therefore, the aims of this study were the following: (1) evaluation of the glycodelin mRNA expression and localization in normal rat lung tissue by RT-PCR and in situ hybridization, (2) evaluation of the glycodelin protein expression and localization in normal rat lung tissue by immunohistochemistry with a specific polyclonal antibody, (3) determination of the potential role of glycodelin in the pulmonary immune response in an experimental rat model of allergen-induced airway inflammation.

Material and methods

Experimental rat model

Male Brown Norway rats (average weight = 200 g) were exposed to a nebulized aerosol of an ovalbumin suspension (10 mg/ml ovalbumin in 0.9% NaCl) as described previously (Kasper et al. 2002), to induce an airway response. Two groups of rats (inhalation of ovalbumin or saline; $n = 5$–7) were challenged for 28 days and whole lungs were removed, fixed with 4% formaldehyde then embedded in paraffin for in situ hybridization and immunohistochemistry or frozen in liquid nitrogen for real-time RT-PCR.

RT-PCR

Total RNA was isolated using guanidinium-isothiocyanate (RNeasy Mini Kit, Qiagen, Hilden, Germany) and RNA concentration was determined by UV absorbance measurements. An amount of 200 ng total RNA was reverse transcribed using random hexamer primers and the TaqMan Reverse Transcription Reagents (PE Applied Biosystems, Weiterstadt, Germany).

PCR was performed with Taq Polymerase (Qiagen) for 40 cycles using a Thermal Cycler P × 2 (Thermo Electron, Dreieich, Germany). The cDNA was amplified with glycodelin-specific primers as described previously (PP14-for1: 5′-CCGCTAGCTAAAGCTTAGCCATGCTGTGCCTCCT GCTCA-3′, PP14-Exon1-rev: 5′-CCGATCACTTCTCG AGCTTTGGGAGCTCCAGGTCCTGCTTGGTC-3′; Keil et al. 1999). For standard RT-PCR an amount of 8 ng reverse transcribed RNA was used in a 50-µl reaction

volume. The thermocycler was programmed to apply an initial cycle consisting of 94°C denaturation for 5 min, followed by 40 cycles of 68°C annealing for 15 s, 72°C elongation for 30 s and 94°C denaturation for 15 s. A final elongation step at 72°C for 5 min was included. A "no-template control" with water was performed alongside all experiments. The obtained PCR product was cloned into the pGEM-T-Easy vector (Promega) using standard procedures. The sequence was determined using the BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Applera Deutschland GmbH) and an automated DNA sequencer. Specificity was verified by NCBI Blast (www.ncbi.nlm.nih.gov/BLAST/).

Preparation of riboprobes

A 227-bp fragment of the glycodelin cDNA (position +41 to +268) was cloned into the *Eco*R1 restriction site of pBluescript SK^- (Stratagene, Germany) and labelled with digoxigenin (DIG) by in vitro transcription using the *DIG RNA labelling Kit* (SP6/T7; Roche Biochemicals, Germany) as described previously (Keil et al. 1999). The antisense cRNA was used for the detection of glycodelin mRNA whereas the sense cRNA probe served as a negative control.

In situ hybridization

Non-radioactive in situ hybridization was performed with paraffin sections (4 μm) which had been fixed in 4% paraformaldehyde. Sections were rehydrated and permeabilized by pepsin digestion (750 μg/ml pepsin in 0.2 M HCl, 37°C, 30 min). Post-fixation (paraformaldehyde 4%, 20 min, 4°C) was followed by acetylation using 0.4% acetic anhydride in triethanolamine (0.1 M, pH 8.0, 15 min). After washing with 50% formamide in 1.5% SSPE (20× SSPE: 3.6 M NaCl, 0.2 M NaH_2PO_4, 0.2 M EDTA, pH 7.4) the sections were prehybridized for 1 h at 56°C in a solution containing 50% formamide and 50% solution D (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0), 0.5% blocking reagent (Roche Biochemicals, Germany) and 210 μg/ml t-RNA. After hybridization for 12–16 h with prehybridization solution containing 125 ng DIG-labelled cRNA probe and washing with 2× SSC (20× SSC: 3 M NaCl, 0.3 M sodium citrate; pH 7.4) sections were incubated with blocking reagent (Roche Biochemicals, Germany). Bound riboprobe was visualized by incubation with alkaline phosphatase-conjugated anti-DIG antibody (Roche Biochemicals, Germany) and subsequent substrate reaction containing 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue-tetrazolium chloride. A blind test was conducted at the same time using identical staff, equipment and chemicals to determine the level of mRNA. From each section, five digital pictures were taken at random of different places of the tissue (200-fold magnification; 3CCD colour camera; Hitachi HV-C20M; Hitachi Denshi Ltd, Japan, and Axiolab, Carl Zeiss, Göttingen, Germany). For standardization of the measurement in each picture the optical density of white background colour was attuned to 250. For all sections we assessed the mean optical density and the quantity of pixels that had a positive reaction for glycodelin by use of KSRun software (imaging system KS400, release 3.0; Zeiss, Vision GmbH, Munich, Germany).

Immunohistochemistry

Indirect immunohistochemistry was performed on paraffin sections (4 μm) using a polyclonal anti-glycodelin antibody (Kunert-Keil et al. 2005) and a biotinylated secondary anti-rabbit antibody (Vectastain® ABC Kit; Vector Laboratories, Burlingame). Sections were incubated in methanol/H_2O_2 (30 min) to inhibit endogenous peroxidase activity, washed in PBS and treated with goat serum (Vectastain® ABC Kit, 20 min, 22°C) to reduce non-specific background staining. Incubation with the polyclonal anti-glycodelin antibody (whole serum; 1:500 in PBS; Bioscience AG) was done overnight at 4°C. Sections were then incubated with the biotinylated secondary goat-anti-rabbit antibody (Vectastain® ABC Kit, 1 h, 22°C) and avidin-biotinylated peroxidase (Vectastain® ABC Kit, 45 min, 22°C). Peroxidase staining reaction was done with diaminobenzidine/H_2O_2 (1 mg/ml; 5 min) and stopped in tap water. Sections were counter stained in haematoxylin and then cover-slipped. In controls, the primary antibody was replaced with pre-immune serum of the respective rabbit. A blind test was conducted at the same time using identical staff, equipment and chemicals to determine the level of antigen. From each section, five digital pictures were taken at random of different places of the tissue (200-fold magnification; 3CCD colour camera; Hitachi HV-C20M; Hitachi Denshi Ltd, Japan, and Axiolab, Carl Zeiss, Göttingen, Germany). For standardization of the measurement in each picture the optical density of white background colour was attuned to 250. For all sections we assessed the mean optical density and the quantity of pixels that had a positive reaction for glycodelin by use of KSRun software (imaging system KS400, release 3.0; Zeiss, Vision GmbH, Munich, Germany).

Results

Detection of glycodelin in rat lung

Standard RT-PCR using the PP14-for1 and PP14-Exon1-rev primers and cDNA from rat lung, a fragment of the first glycodelin exon was obtained with the expected size of

about 100 bp (position +41 to +140; Fig. 1a, b). The sequence of the subcloned rat lung glycodelin fragment revealed 100% homology at the nucleotide level with the human mRNA sequence of the PAEP (placental protein 14; accession number: NM_002571; Fig. 1c).

Ovalbumin-induced increase in glycodelin expression

In situ hybridization with the glycodelin antisense cRNA probe revealed a strong signal in cells of the bronchial epithelium, e.g. Clara cells and Goblet cells in normal and ovalbumin-treated rat lungs (Fig. 2a). In alveolar epithelial cells, particular type II pneumocytes and alveolar macrophages glycodelin-specific transcripts could be detected as well. However, the expression levels seemed rather weak. In ovalbumin-treated rat lungs a very strong staining of the glycodelin mRNA was detected in inflammatory cells, e.g. macrophages surrounding bronchioli and blood vessels. All sections incubated with the corresponding sense cRNA probes were devoid of any positive reaction.

Computerized analysis of staining intensity (optical density) is shown in Fig. 2b. We found significant increase in glycodelin mRNA expression in ovalbumin-treated rat lungs compared to control rat lungs ($*P < 0.005$). In addition, there were no significant differences in glycodelin mRNA expression in rat left lungs and its corresponding right lungs of both experimental groups (data not shown).

In both groups, rats after saline or ovalbumin challenge, the glycodelin protein was found to be localized in alveolar epithelial cells, alveolar macrophages and cells of the bronchial epithelium, predominantly in ciliated cells (Fig. 3a). Furthermore, inflammatory cells, e.g. macrophages surrounding bronchioli and blood vessels showed very strong glycodelin immunoreaction in ovalbumin-treated rat lungs. All sections incubated with the pre-immune serum were negative.

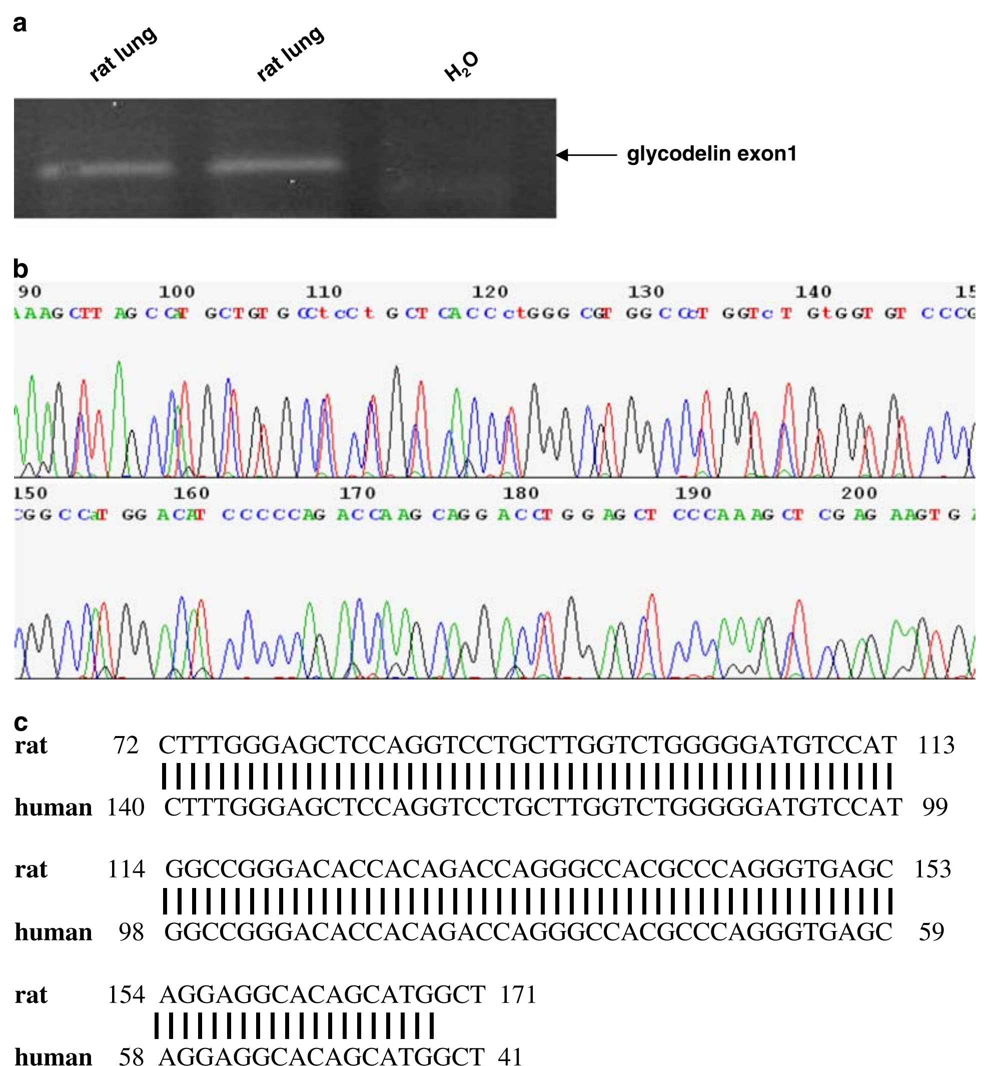

Fig. 1 Expression and sequence analyses of a glycodelin exon 1 fragment from rat lung. **a** RT-PCR; Reverse transcribed RNA (8 ng) from rat lung tissue was added to the reaction mixtures and PCR products amplified in 40 cycles. The products from two different rat lung tissue specimens were separated on agarose gels and stained with ethidium bromide. Water served as negative control. **b** Sequence chromatogram; DNA was sequenced using the BigDye® terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) and the ABI Prism 310 Sequencer Analyzer. **c** Alignment of the glycodelin exon 1 fragment from rat lung with the human mRNA sequence of the progestagen-associated endometrial protein (placental protein 14; accession number: NM_002571)

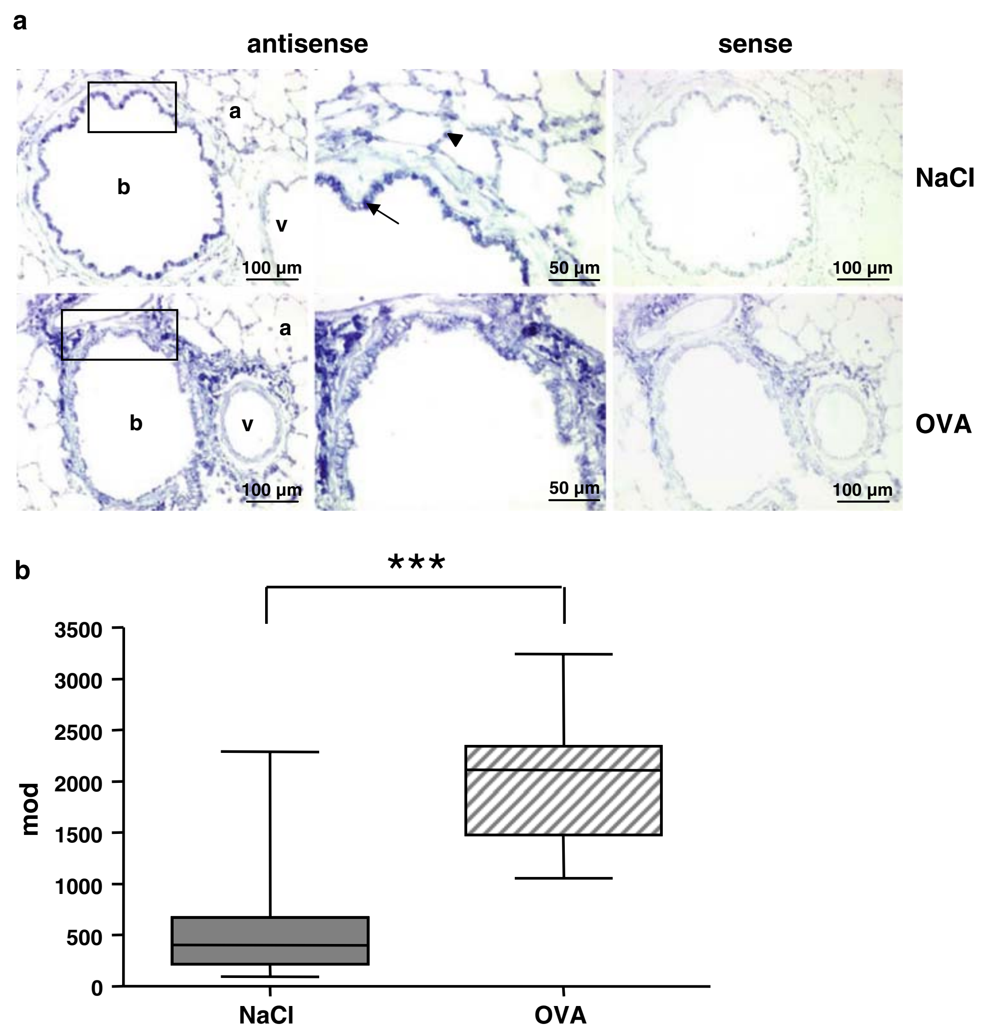

Fig. 2 In situ hybridization and densitometric analyses of glycodelin mRNA in control and ovalbumin-treated rat lungs. **a** Glycodelin mRNA distribution in control and ovalbumin-treated rat lungs was determined by in situ hybridization using digoxigenin-labelled glycodelin antisense cRNA probe. The *arrow* indicates a Clara cell, the *triangle* shows a type II pneumocyte. *a* alveole, *b* bronchioli, *v* blood vessel. **b** Densitometric analyses of the in situ hybridization experiments using the KSRun software. Mean ± SEM are given for $n = 5$–7 animals. Student's *t*-test (***$P < 0.005$); mod = mean optical density; *OVA* ovalbumin

The glycodelin antigen expression determined by densitometric analyses of the immunohistochemistry was increased twofold in the ovalbumin challenge group compared to the levels of glycodelin in rat lungs after saline challenge (*$P < 0.005$, Fig. 3b). Like glycodelin mRNA there were no significant differences in glycodelin protein expression in rat left lungs and its corresponding right lungs of both experimental groups (data not shown).

Discussion

In the present study glycodelin, which was formerly predominantly detected in tissues of the female and male genital tract, was shown for the first time to be expressed in lung tissue of rats. These findings strongly suggest that Gd expression is of functional relevance in the lung of mammals. In the present study it turned out that Gd is localized in type II pneumocytes and cells of the bronchial epithelium. These results are in accordance with those of Kämäräinen et al. (1997), who demonstrated the localization of Gd in highly differentiated acinar epithelia, e.g. sweat glands, breast and pancreatic cystadenoma. Therefore, it can be speculated that Gd expression is related to differentiated epithelium. On the other hand, in vitro studies on the human breast carcinoma cell line MCF-7 revealed that Gd expression in these cells is accompanied by the acquisition of an organized glandular epithelium (Kämäräinen et al. 1997). This may indicate Gd as a marker of epithelial differentiation. The induction of Gd expression has previously been used to assess differentiation in endometrial epithelial cells in vitro (Arnold et al. 2001). Furthermore, recent work has shown that epithelial cells are capable of producing and responding to a number of immunomodulatory peptides, such as glycodelin (Laird et al. 1993). Furthermore, the present study demonstrates the presence of glycodelin in alveolar macrophages and inflammatory cells, e.g. macrophages surrounding bronchioli and blood vessels. In leiomyoma, glycodelin co-localized also with macrophages shown by immunohistochemistry with the macrophage antibody HAM-56 (Ramachandran et al. 2005).

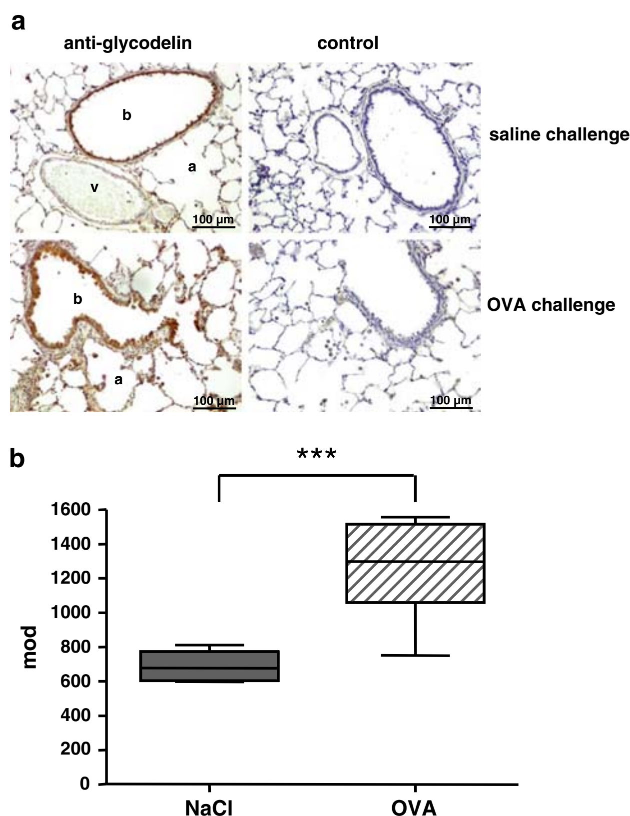

Fig. 3 Immunohistochemistry and densitometric analyses of glycodelin in control and ovalbumin-treated rat lungs. **a** Glycodelin protein distribution in control and ovalbumin-treated rat lungs was determined by indirect immunohistochemistry using a polyclonal anti-glycodelin antibody and a biotinylated secondary anti-rabbit antibody. *a* alveole, *b* bronchioli, *v* blood vessel. **b** Densitometric analyses of the immunohistochemical experiments using the KSRun software. Mean ± SEM are given for $n = 5$–7 animals. Student's *t*-test (***$P < 0.005$); mod = mean optical density; *OVA* ovalbumin

Expression of glycodelin is believed to be related to the action of steroids because the Gd gene contains three putative progesterone/glucocorticoid regulatory elements, of which one is located in the promoter region (Vaisse et al. 1990). A hormonal regulation of the expression of Gd was shown by progesterone. Glycodelin production by endometrial cells was directly up-regulated fourfold to ninefold in vitro (Taylor et al. 1998). Furthermore, studies have indicated an association between follicular-phase serum estradiol levels and luteal-phase serum glycodelin concentration (Li et al. 1992). However, transcriptional regulation of the glycodelin promoter is not oestrogen dependent (Taylor et al. 1998, 2000). In contrast to these findings in humans the prevalence of asthma is higher among females than among males after puberty. The biological sex differences include genetic, pulmonary and immunological factors. Melgert et al. (2005) found that female mice develop a more pronounced type of allergic airway inflammation than male mice after ovalbumin challenge. Female PBS treated mice had significantly lower percentages of regulatory $CD4^+/CD25^+$ T cells than males (Melgert et al. 2005).

In our study the gender differences in asthma development and progression were avoided because only male rats were included in both experimental groups. Even so OVA treated male rats with allergic airway inflammation showed significant higher glycodelin expression compared to saline treated rats. The function of glycodelin in the lung is not clear. Since glycodelin expression increased during the development of allergic asthma in OVA treated rats a role in immune response seems to be possible. A malfunction of the adaptive immunity is one possible reason for allergic asthma. Asthma is characterized by airway inflammation dominated by the presence of eosinophils and $CD4^+$ T lymphocytes (Busse and Lemanske Jr 2001). The pulmonary $CD4^+$ T cells produce predominantly high amounts of Th2-type cytokines, such as IL-4, IL-5, IL-9 and IL-13 with subsequent increased production of allergen-specific IgEs (Holt et al. 1999). Enhanced ovalbumin-specific IgE levels were shown in the same rat model as used for our study (Kasper et al. 2002). Akbari et al. (2006) could further show that $CD4^+$ natural killer T cells play a prominent pathogenic role in human asthma. Glycodelin has immunosuppressive properties. Glycodelin inhibits natural killer cell activity (Okamoto et al. 1991). Immuno-purified Gd from first trimester amniotic fluid has been reported to function as a direct inhibitor of T cell proliferation (Rachmilewitz et al. 1999) and an inducer of T cell apoptosis (Rachmilewitz et al. 2001; Mukhopadhyay et al. 2004). Furthermore, it was shown that GdA causes inhibition of G1/S progression together with up-regulation of cyclin-dependent kinase inhibitors including p21, p27 and p16 thereby reducing cell proliferation activity in endometrial epithelial cells (Ohta et al. 2008). The increased Gd expression could be a compensatory effect of lung damage. It is to speculate that all these functions of glycodelin could cause in a return to normal of the immune response in the lung.

Another aspect in the development of allergic airway inflammation is the mucous hypersecretion, which is a complex event involving goblet-cell hyperplasia, Th2 cytokine driven mucous production involving Clara and other lung cells (Kuperman et al. 2005), and increased surfactant production from type II pneumocytes. In this case, the impact of glycodelin as Th1 response inhibitor (Mishan-Eisenberg et al. 2004) has to be considered.

Thirdly, because glycodelin is known to function as a differentiation marker in epithelial cells (Kämäräinen et al. 1997; Arnold et al. 2001) the regeneration of the destroyed tissue in allergic asthma can cause in an increase of the glycodelin expression. Airway fibrosis and damage of the epithelium including goblet-cell hyperplasia (airway wall remodelling) are characteristic features of chronic asthma.

Mice treated with ovalbumin showed marked peribronchial fibrosis (Koerner-Rettberg et al. 2008).

In addition, asthma has been described to increase the risk of adverse maternal pregnancy outcomes like pre-eclampsia and placenta previa (Demissie et al. 1998). Recently was shown that the expression of glycodelin was significantly reduced in pre-eclamptic, HELLP and intra-uterine growth-restricted decidual tissue (Jeschke et al. 2005a).

The course of asthma can be changed by pregnancy in variable ways for unknown reasons. An equal number of women have asthma symptoms that improve, worsen, or are unchanged through pregnancy. If symptoms worsen, it usually occurs in the second and third trimesters, with the peak in the sixth month (Gluck and Gluck 2006). Taken together those findings, glycodelin might improve asthma symptoms in the first trimester of pregnancy. Reduced glycodelin production and immunosuppressive action in the second and third trimester of pregnancy together with additional risk factors like pre-eclampsia may worsen asthma symptoms.

In summary, glycodelin expression is increased in ovalbumin-treated rat lungs. Increased glycodelin expression could be accompanied with increased autoimmune mechanism on one hand but also with the regeneration of destroyed bronchial and alveolar epithelium tissue and compensatory effects of lung damage on the other hand.

Acknowledgements We thank D. Schulz and H. Kenk for excellent technical assistance. Furthermore, we are grateful to Terry Shaw (former correspondent to the Daily Telegraph) for his support.

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