Original Article

Early Stages of Trachea Healing Process: (Immuno/Lectin) Histochemical Monitoring of Selected Markers and Adhesion/Growth-Regulatory Endogenous Lectins

(wound healing / tissue repair / regeneration / galectin / glycophenotype / tracheotomy)

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Abstract. Tracheotomy may be associated with numerous acute and chronic complications including extensive formation of granulation tissue. The emerging functional versatility of the adhesion/growth-regulatory galectins prompted us to perform a histochemical study of wound healing using rat trachea as model. By using non-cross-reactive antibodies and the labelled tissue lectins we addressed the issue of the presence and regulation of galectin reactivity during trachea wound healing. Beside localization of high-molecular-weight keratin, wide-spectrum cytokeratin, keratins 10 and 14, α-smooth muscle actin, vimentin, fibronectin, and Sox-2, galectins -1, -2, and -3 and their reactivity profiles were measured in frozen sections of wounded and control trachea specimens 7, 14, and 28 days after trauma. A clear trend for decreased galectin-1 presence and increased reactivity for galectin-1 was revealed from day 7 to day 28. Sox-2-positive cells were present after seven days and found in the wound bed. Interestingly, several similarities were observed in comparison to skin wound healing including regulation of galectin-1 parameters.

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Abbreviations: Gal – galectin, HE – haematoxylin-eosin, M/TM – monocytes/tissue macrophages, PBS – phosphate-buffered saline, PMNL – polymorphonuclear leukocytes, VG – Van Gieson.

Introduction

Tracheotomy may be followed by numerous acute and chronic complications. The most frequent reaction manifested late as response to the surgical procedure is the development of excess granulation tissue. Its presence may lead to airway occlusion. Such a reaction may occur in up to 65 % of the patients (Wood and Mathiesen, 1991; Sue and Susanto, 2003), requiring prompt treatment. Several studies have been published pointing to an impact of various factors within the management of tracheal stenosis (Talas et al., 2002; Liman et al., 2005; Sarper et al., 2005; Herrington et al., 2006).

It is generally accepted that the key problems of trachea healing are associated with extensive formation of granulation tissue. In detail, this process leads to hypertrophic scar formation and may finally result in tracheal

stenosis (Talas et al., 2002). Different techniques have been developed and successfully used to treat tracheal stenosis, such as implementation of stents (Fernando et al., 2010). However, these techniques are rather time- and money-consuming and they cause additional stress for the patient because further surgical interventions are often inevitable. Hence, intervention by medications favouring trachea wound healing and preventing stenosis formation would reduce costs and be beneficial for patients.

At the molecular level, growth factors as well as chemo- and cytokines are known to play significant roles in the coordinated events leading to complete post-traumatic tissue repair and regeneration (Gomperts et al., 2007; Sandulache et al., 2009). In addition, increasing attention is given to the role of glycosylation of proteins/lipids, the sugar-encoded information being translated into cellular activities by endogenous lectins (Gabius et al., 2004, 2011). Of note, the presence of distinct endogenous lectins and the presentation of appropriate glycan epitopes on counter-receptors such as the fibronectin receptor are orchestrated in a finely tuned manner to generate potent signals regulating e.g. cell growth (for concept and overview see Gabius, 2009; for model studies on control exerted by a tumour suppressor or within T-cell communication see André et al., 2007; Wang et al., 2009; Sanchez-Ruderisch et al., 2010; Wu et al., 2011). In the mentioned cases, members of the galectin family serve as the lectin part capable to effectively regulate cell adhesion, growth and migration, via protein/glycan and protein/protein interactions (Gabius, 2006; Smetana Jr. et al., 2006; Schwartz-Albiez, 2009; Kaltner and Gabius, 2012). This emerging activity profile prompted us to study the presence of adhesion/growth-regulatory galectins focusing on the homodimeric proto-type galectins -1 and -2 (Gal-1, Gal-2) as well as the chimera-type galectin-3 (Gal-3). Special care was taken to preclude any cross-reactivity in immunodetection, and purified galectins were in parallel applied as sensors for changes in cellular reactivity. Beside application of these tools, formation of the extracellular matrix and the signature of keratin expression were assessed.

Hence, in this investigation, distribution of endogenous lectins and their binding sites were set in relation to keratin expression and extracellular matrix formation. Since the status of sialylation is known to regulate galectin reactivity markedly, with α-2,6-sialylation acting as stop signal except for the presence on termini in repeats (Ahmed et al., 2002), this parameter was additionally monitored by two plant lectins. In view of recent evidence that adult stem cells play a remarkable role in wound healing, the presence of Sox-2, nucleostemin, and vimentin was evaluated as well. Tissue specimens were processed under identical conditions to rigorously exclude any factor other than the time point to affect signal occurrence and intensity.

Material and Methods

Animal model

The study was approved by the local ethical committee and by the State Veterinary and Food Administration of the Slovak Republic.

One-year-old male Sprague-Dawley rats (N = 20) were included into the experiment. In all rats, surgery was performed under general anaesthesia induced by administration of ketamine (40 mg/kg; Narkamon a.u.v.; Spofa, Prague, Czech Republic), xylazine (15 mg/kg; Rometar a.u.v.; Spofa) and tramadol (5 mg/kg; Tramadol-K; Krka, Novo Mesto, Slovenia). Prior to tracheotomy each rat was intubated. Under aseptic conditions, skin and subcutaneous tissue were incised, then strap muscles were separated and retracted laterally to enable access to the front tracheal wall. Next, median incision was performed from the second to the fifth tracheal cartilage ring. Subsequently, the incision was closed using two simple interrupted sutures (Chiraflon 6/0; Chirmax, Prague, Czech Republic), as was done for the wound in anatomical layers (Chiraflon 5/0). Overall, six rats were killed by ether inhalation 7, 14, and 28 days after surgery, respectively. Intact trachea was removed from two control rats, which did not undergo surgery.

Human trachea

A sample of human trachea was obtained from a non-smoking volunteer suffering from tracheal stenosis after punction tracheostomy. The sample was obtained after informed consent of the patient with agreement of the local ethical committee.

Basic histology

Tracheae were removed from one intact rat and four wounded rats killed at each evaluated time point and routinely processed for light microscopy (fixation in 4% buffered formaldehyde, dehydration, paraffin embedding, sectioning, and staining). Paraffin sections were stained with haematoxylin-eosin (HE – basic staining) and Van Gieson (VG – non-specific collagen staining).

Immunohistochemistry and lectin histochemistry

Tracheae were cryoprotected by Tissue-Tek from one intact rat and two wounded rats killed at each given time point (Sakura, Zoeterwoude, The Netherlands) and stored in liquid nitrogen. Frozen sections were fixed in 2% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (pH = 7.2). Non-specific binding of secondary antibody was blocked by preincubation with normal swine serum (DAKO, Glostrup, Denmark) diluted in PBS for 30 min. The origin of the reagents for immunohistochemistry and lectin histochemistry used in this study is listed in Table 1. Purification of galectins, rigorous controls for purity, their labelling under activity--preserving conditions and activity controls as well as preparation of the antibodies, testing for and removal of

Table 1. Reagents used for immunohistochemistry and lectin histochemistry

primary antibody	abbreviation	host	produced by	secondary antibody	produced by	channel
high-molecular-weight cytokeratin	HMWK	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	(Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
vimentin	Vim	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	(both Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
cytokeratin 10	K10	mouse monoclonal	DakoCytomation, Glostrup, Denmark	goat anti-mouse	(both Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
cytokeratin 14	K14	mouse monoclonal	Sigma, Saint Louis, Missouri	goat anti-mouse	(both Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
wide-spectrum cytokeratin	WSK	rabbit polyclonal	Abcam, Cambridge Science, Cambridge UK	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA)	FITC-green
fibronectin	Fibr	rabbit polyclonal	Dakopatts, Glostrup, Denmark	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA)	FITC-green
Sox-2	Sox2	rabbit polyclonal	Abcam, Cambridge Science, Cambridge UK	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA)	FITC-green
galectin 1	Gal-1	rabbit polyclonal	Gabius laboratory	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA)	FITC-green
galectin 2	Gal-2	rabbit polyclonal	Gabius laboratory	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA)	FITC-green
galectin 3	Gal-3	rabbit polyclonal	Gabius laboratory	swine anti-rabbit	(Santa Cruz Biotechnology, Santa Cruz, CA)	FITC-green
biotinylated lectin	**abbreviation**		**produced by**	**second step reagent**	**produced by**	**channel**
galectin 1 binding site	Gal-1BS		Gabius laboratory	ExtrAvidin	(Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
galectin 2 binding site	Gal-2BS		Gabius laboratory	ExtrAvidin	(Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
galectin 3 binding site	Gal-3BS		Gabius laboratory	ExtrAvidin	(Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
Maackia amurensis (α2,3-linked NeuNAc)	MAA		Vector Laboratories, Burlingame, CA	ExtrAvidin	(Sigma-Aldrich, Prague, Czech Republic)	TRITC-red
Sambucus nigra (α2,6-linked NeuNAc)	SNA		Vector Laboratories, Burlingame, CA	ExtrAvidin	(Sigma-Aldrich, Prague, Czech Republic)	TRITC-red

any cross-reactivity among galectins and quality controls by Western blotting/ELISA were performed as described previously (Gabius et al., 1991; Purkrábková et al., 2003). All antibodies were routinely tested against galectins -1, -2, -3, -4, -5, -7, -8, and -9 (galectin-6 is restricted in occurrence to several mouse strains). Nuclei were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO). Specimens were mounted to Vectashield (Vector Laboratories, Burlingame, CA). The analysis of specimens and data storage were performed using an Optiphot-2 fluorescence microscope equipped with filter blocks specific for DAPI, FITC, and TRITC (Nikon, Tokyo, Japan), a CCD camera (COHU) and computer-assisted image analysis system LUCIA 3.2 (Laboratory Imaging, Prague, Czech Republic), as described for nucleostemin monitoring previously (Čada et al., 2007).

Morphometric and semi-quantitative evaluation of histological sections

The area covered by granulation tissue was determined in a section obtained from each individual animal, based on VG staining. The specimen analysis was performed using an Olympus BX51 microscope (Olympus, Tokyo, Japan) with an Olympus DP71 CCD camera and QuickPHOTO MICRO 2.2 (Promicra, Prague, Czech Republic) software. Data are expressed as mean ± standard deviation. A semi-quantitative method (Grendel et al., 2011) was used to evaluate re-epithelization, the presence of inflammatory cells (polymorphonuclear leukocytes [PMNL]), the presence of chondroblasts and of newly formed vessels. Sections were examined as coded slides by two well-trained experts according to the scale ranging from 0 to 3 (Table 2). Similarly, signal intensi-

Table 2. Scale for the semi-quantitative evaluation of histological parameters in healing trachea

Scale	re-epithelization	PMNL	new vessels	new collagen	new cartilage	fluorescence signal
–	absent	absent	absent	absent	absent	absent
+	migration of cells	mild	mild	mild	minimal	mild
++	bridging incision	moderate	moderate	moderate	moderate	moderate
+++	complete regeneration	marked	marked	marked	marked	marked

Table 3. Results from the semi-quantitative evaluation of galectin expression/reactivity

	Gal-1	Gal-1BS	Gal-2	Gal-2BS	Gal-3	Gal-3BS	MAA	SNA
HT	+	+	++	+	++	+	–	+
RT	+	+	++	+	++	+	–	++
7d	++	+	++	++	+	+	–	++
14d	+	+	+++	++	+	++	–	++
28d	+	++	++	++	+	+	–	++

(HT – human trachea, RT – rat trachea)

Table 4. Results from the semi-quantitative evaluation of histological structures/changes during trachea wound healing

	re-epithelization	PMNL	fibronectin	collagen	new cartilage
7d	++	+	+++	–	–
14d	+++	–	++	–	+
28d	+++	–	+	+	++

ties of the galectin presence and reactivity were assessed by a semi-quantitative method according to this scale (Table 2) as previously described in detail (Čada et al., 2009).

Results

During the post-surgical period all animals remained healthy, without any clinical symptoms of infection. The data of the semi-quantitative analysis of histological sections are summarized in Table 3 and Table 4. A detailed account of the obtained results is given for each time point as follows.

Intact trachea

Similar glyco- and immunophenotypes were observed for both human and rat tracheae (Fig. 1, Fig. 2). In contrast to the wide-spectrum cytokeratin antibody, which stained the entire epithelial population, the expression of high-molecular-weight keratin was detectable in a rather limited population of cells (Fig. 2). Similarly, keratin 14 positivity was restricted to the non-ciliated cell population (Fig. 2), whereas keratin 10-positive cells were not found in the tracheal epithelium (not shown). Control trachea was positive for the plant lectin reactive with α-2,6-sialylation, whereas no α-2,3-sialylation on type II termini (i.e. Gal β1, 4GlcNAc) was

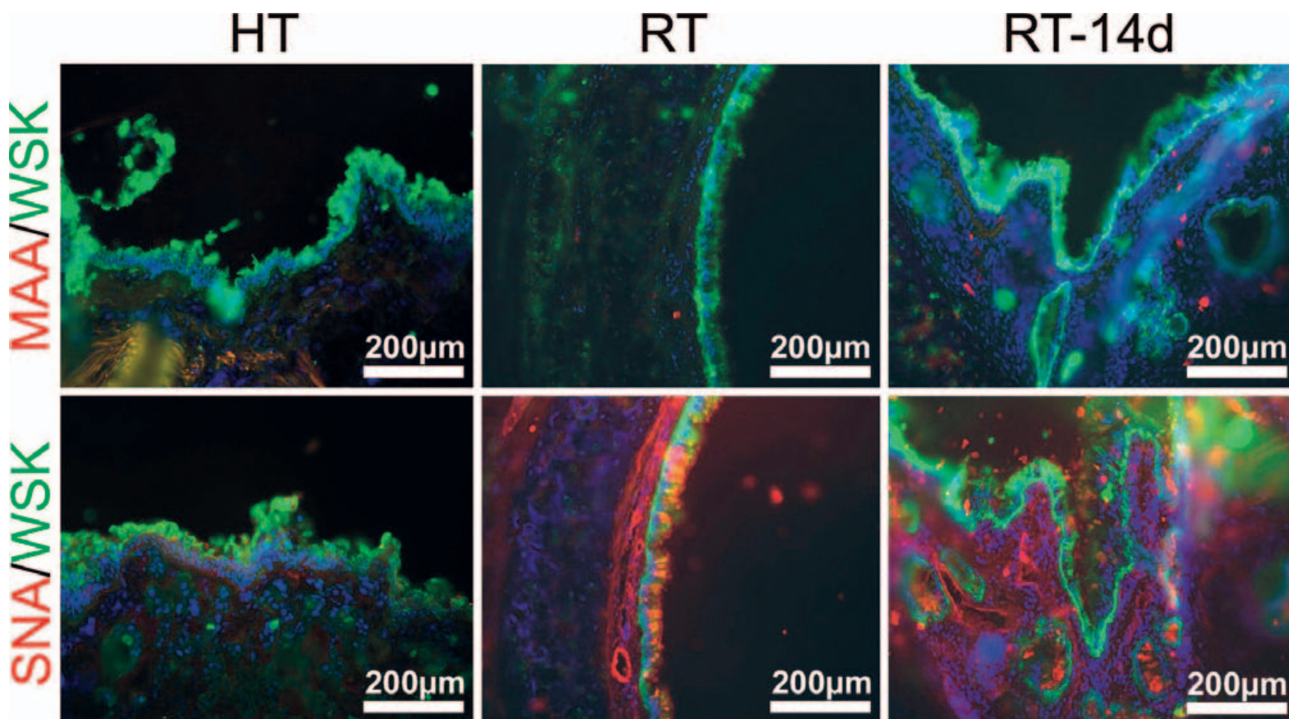

Fig. 1. Binding patterns of the two plant lectins selective for α-2,3- and α-2,6-sialylation in control and injured trachea: a – control trachea/b – wounded trachea 14d post surgery – lack of MAA reactivity under experimental conditions; c – control trachea/d – wounded trachea – SNA reactivity; 200×.

a	b	c
d	e	f

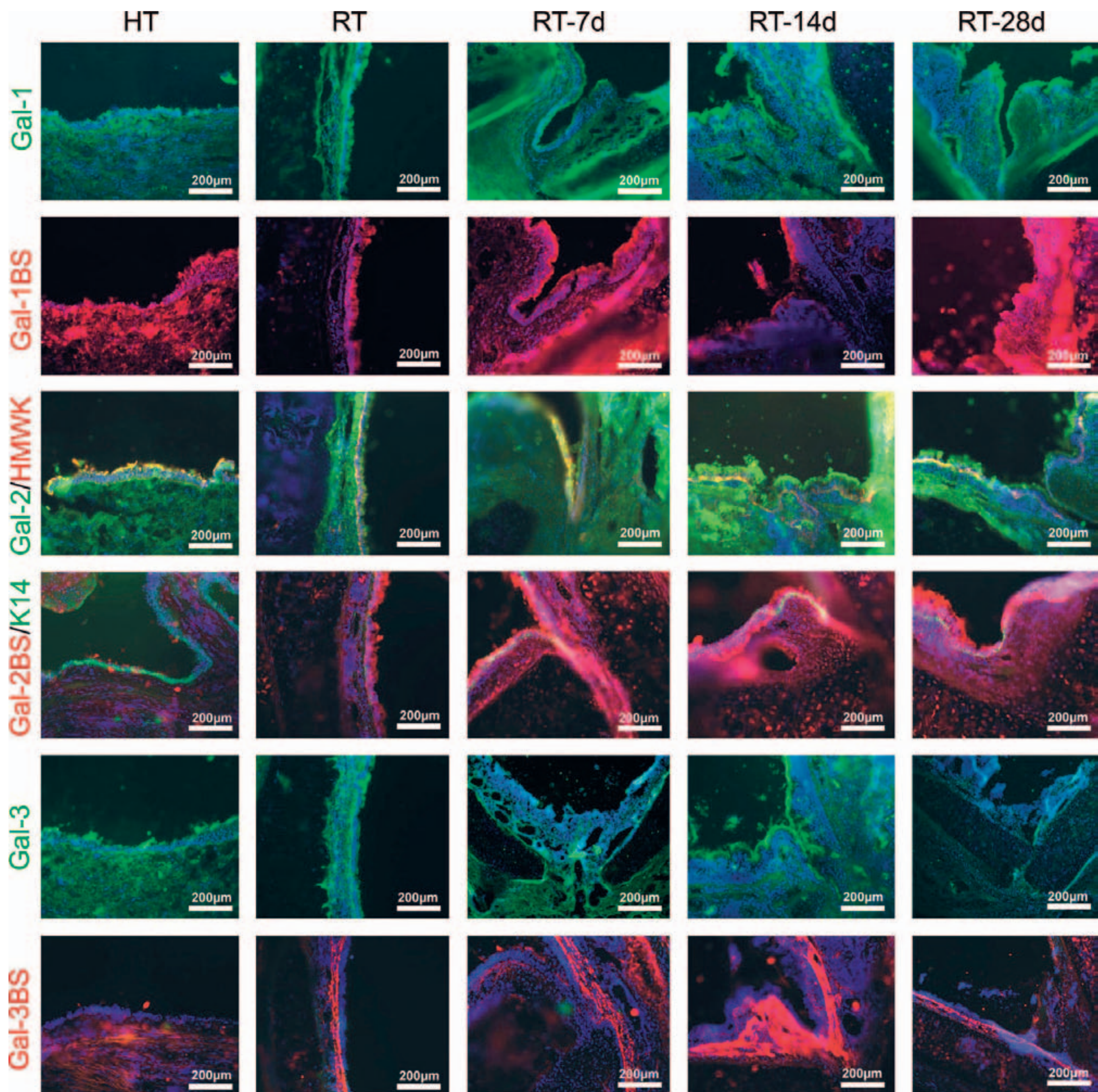

Fig. 2. Galectin (immuno)histochemistry of control and injured tracheae: control (intact) human trachea – a, f, k, p, u, and z; control (intact) rat trachea – b, g, l, q, v, and a1; trachea 7d post surgery – c, h, m, r, w, and b1; trachea 14d post surgery – d, i, n, s, x, and c1; trachea 28d post surgery – e, j, o, t, y, and d1; 200×.

a	b	c	d	e
f	g	h	i	j
k	l	m	n	o
p	q	r	s	t
u	v	w	x	y
z	a1	b1	c1	d1

detectable (Fig. 1). The presence of galectins and their binding sites was as follows (Fig. 2): moderate staining intensity for galectins was seen in the epithelium and in subepithelial tissue. The cartilage and the chondrocytes did not reveal any signal for any of the tested galectins. The binding patterns for Gal-1 and -2 were similar and were located both in the epithelium and in the subepithelial tissue, with moderate up-regulation of Gal-1 on day 7 following injury. In contrast, binding sites for Gal-3 were strictly confined to a thin layer of the subepithelial tissue.

7d post surgery

At this time point, the epithelial sheet completely bridged the incision (Fig. 3). Well-formed granulation tissue was present in wounds removed on day 7 (granulation tissue area = 87.925 ± 21.547 μm^2). Near the site of injury, a slight increase of fibronectin deposition was seen (Fig. 3). No α-smooth muscle actin was detectable (not shown). Keratin 14-positive cell populations were present in the tracheal epithelium (Fig. 2). No keratin 10-positive cells were seen in the epithelium (not

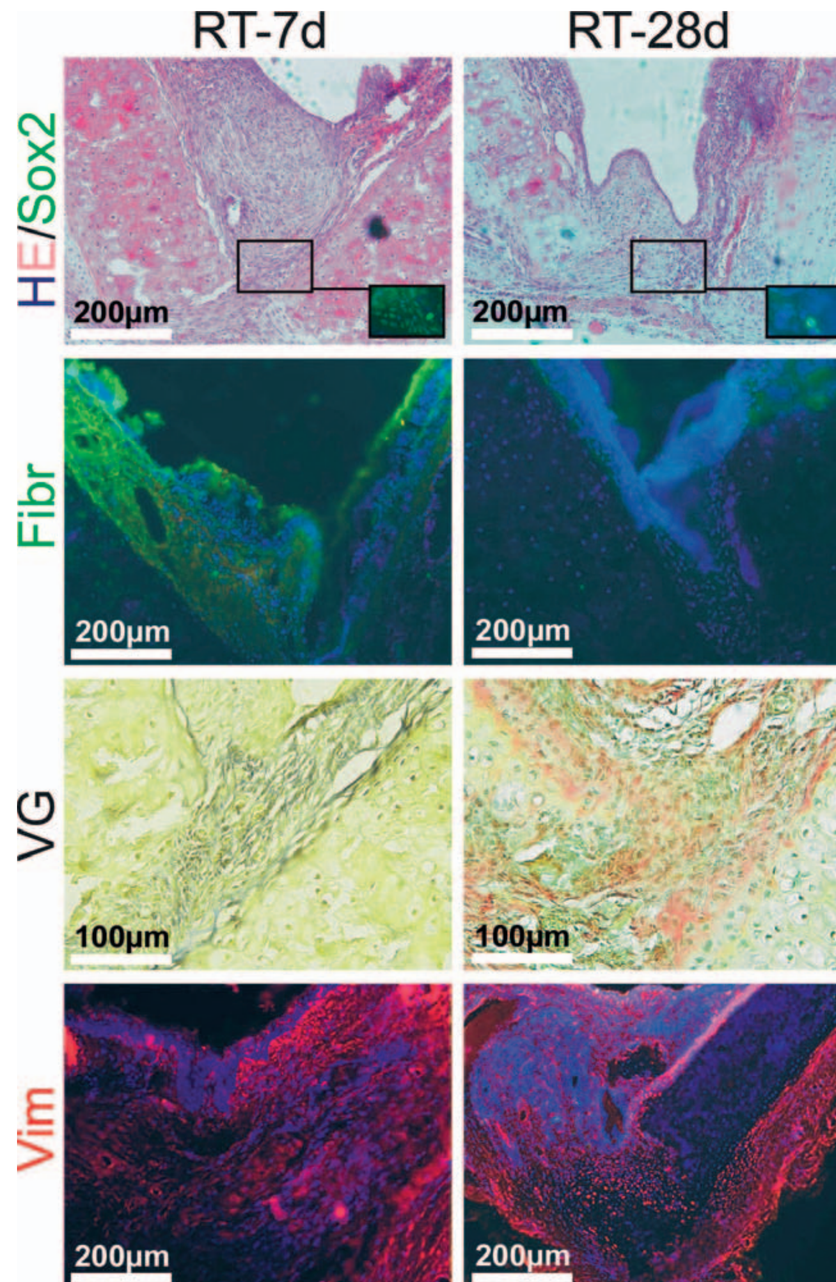

Fig. 3. Wounded trachea: a – 7d post surgery (HE (200×) – staining, well-formed granulation tissue, insert – Sox-2-positive cells in the wound bed); b – 28d post surgery (HE (200×) – staining, reduced granulation tissue, insert – reduced number of Sox-2-positive cells in the wound bed); c – 7d post surgery (fibronectin staining (200×), presence of fibronectin at the injury site); d – 28d post surgery (fibronectin staining (200×), absence of fibronectin at the injury site); e – 7d (VG (400×) – staining, absence of collagen at the injury site); f – 28d post surgery (VG (400×) – staining, presence of collagen at the injury site); g – 7d post surgery (vimentin-positive cells in the granulation tissue (200×)); h – 28d post surgery (presence of vimentin-positive cells (200×), formation of new cartilage).

a	b
c	d
e	f
g	h

shown). Moreover, several Sox-2-positive cells were found in the wound bed (Fig. 1 – insert). Monitoring galectin presence revealed a marked level of Gal-1 expression at the site of injury; Gal-2 was seen in the epithelium and subepithelial tissue of the trachea (Fig. 2). Gal-3 staining was weak, present in the subepithelial tissue and epithelium (Fig. 2). Moderate signals were obtained for binding of labelled Gal-1 and -2 in the epithelium and subepithelial tissue, Gal-3 reactivity was maintained in a thin layer of the subepithelial tissue (Fig. 2). As seen in control trachea, evidence for α-2,6--sialylation was obtained at the site of injury (not shown).

14d post surgery

The number of PMNL remained unchanged, while the number of monocytes/macrophages increased when compared to sections removed on day 7 (not shown). The largest number of new vessels in the granulation

tissue was observed at this time point (not shown). Its area decreased to 75.591 ± 8.878 μm^2. The content of fibronectin also decreased in the granulation tissue, whereas the collagen content increased (not shown). Furthermore, the number of Sox-2-positive cells decreased (not shown). Positivity for Gal-1 and -2 was maintained in the epithelium and subepithelial tissue (Fig. 2). There was no signal for the Gal presence in the cartilage. Mild reactivity for Gal-1 was determined, Gal-3 binding slightly increased (Fig. 2). The profile for α-2,6-sialylation remained unchanged 14 days post-wounding (Fig. 1).

28d post surgery

A month after surgery, the remodelling/maturation phase of healing was still in progress (Fig. 3). The number of newly formed vessels decreased, together with the overall area of granulation tissue (67.612 ± 10.612 μm^2). The number of PMNL reached its minimum, whereas the number of monocytes/macrophages remained rather moderate (not shown). Moreover, the density of fibronectin in the granulation tissue also decreased to its minimum, while the content of collagen continued to increase (Fig. 3). On the other hand, the number of Sox-2-positive cells was diminished (Fig. 1 – insert). As already noted above, a concomitant reduction in Gal-1 was seen (Fig. 2). Conversely, the signal intensity for binding sites of this lectin reached its highest level (Fig. 2). Binding of Gal-2 and -3 followed a constant pattern. In addition, reactivity for Gal-2 was also observed in the chondroblasts of the cartilage (Fig. 2). No change in the α-2,6-sialylation was seen (not shown).

Discussion

The range of physiological activities of endogenous lectins prompted us to systematically determine the presence of three adhesion/growth-regulatory galectins at three time points post-trauma and in uninjured control tissue, along with several known marker proteins. According to our observations complete wound covering with a layer of epithelial cells was already seen on day 7. Most epithelial cells were positive for keratin 14, but all cells were negative for keratin 10. The epithelial cells expressed Gal-1, -2 and -3. Interestingly, high-molecular-weight keratin antibody did not stain any cell; its presence was rather seen in the basal layer of the cylindrical epithelium and not in ciliated cells, similar to the positivity profile for keratin 14.

Our experiments with non-cross-reactive antibodies and biotinylated galectins answered the questions on the level of presence, regulation in the course of healing and inter-galectin differences. Galectin presence could be detected in all cases. By testing two plant lectins no evidence for a change in the sialylation status, a decisive parameter in T-cell activation and tumour suppressor activity controlling galectin reactivity (André et al., 2007; Bi and Baum, 2009; Sanchez-Ruderisch et al., 2011), was detected. However, applying the tissue lectins as tools, different profiles among the tested galectins were observed during the course of the repair processes. Both localization patterns and signal intensity were clearly disparate. Despite the overall sequence homology, each protein tested followed its characteristic course and, of note, regulation within the healing process. Most prominently, Gal-1 appeared to be up-regulated in the early phase of tissue repair and regeneration, then leveled off, with matching changes in tissue reactivity for Gal-1. Co-regulation has been observed to direct e.g. anoikis induction by a tumour suppressor as master regulator (André et al., 2007). The changes in Gal-1 and -3 were similar when compared to rat skin wound healing (Gál et al., 2011). Obvious differences among galectins give research a clear direction into deciphering mechanisms underlying gene regulation, based on the results of mining proximal promoter regions for putative sites binding transcription factors (Sturm et al., 2004; Lohr et al., 2007, 2008). This conclusion is supported by the finding of disparate profiles also in wound healing of pig skin (Klíma et al., 2009).

A peak in the proliferation phase among the evaluated time intervals of trachea healing was observed on day 7. At this time point, an increased number of vessels and vimentin-positive cells was observed, suggesting an involvement in such parameter changes. When compared to skin wound healing in rats, there is a slight delay for these alterations to occur (Gál et al., 2008). Together with the increase in the number of cells, the collagen content in the trachea wound was elevated. Of note, the contents of fibronectin and collagen had an inverse relationship. This relationship is necessary for the sufficient biomechanical stiffness of the injured tissue to be accomplished. On the other hand, hyperproliferation of granulation tissue might result in collagen overproduction. Later on, a hypertrophic scar can be formed, a manifestation often underlying airway occlusion (Liman et al., 2005).

Sox-2 is an evolutionarily conserved transcription factor that plays an important role in oesophagus and trachea development (Que et al., 2009). In that study, it was shown that Sox-2 impairs proliferation and differentiation of adult tracheal epithelium during regenerative processes. In our histological study, we demonstrated that Sox-2 was expressed in the cells that form an endoluminal wound bed of healing trachea, and its expression decreased with healing time. From this point of view, it may be suggested that – in addition to epithelial stem cells – a limited population of adult stem cells is located in the perichondrium and wound bed and that these cells might contribute to the formation of granulation tissue during trachea wound healing.

In summary, our study characterized the galectin presence and reactivity in the course of healing of tracheal incisional wounds in the rat model. The detected differences argue in favour of functional individuality and also intracellular activities of the tested proteins. Since the sialophenotypes of intact human and rat tra-

chea were similar, it may be suggested that the rat model might have relevance for the clinical situation. Consequently, analysis of clinically relevant specimens following this concept is now warranted.

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