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Immunohistochemical profile of galectin-8 expression in benign and malignant tumors of epithelial, mesenchymatous and adipous origins, and of the nervous system

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Summary. This study aims to investigate whether the immunohistochemical expression of galectin-8 could be used as a diagnostic marker in tumor tissues of various histogenetic origins including specimens from epithelial (n=145), mesenchymatous (n=16), adipous (n=10) and central and peripheral nervous system (n=25) tissue, and 4 mesotheliomas. Immunohistochemical reactions were carried out with a polyclonal anti-galectin-8 antibody and histological slides from tissues derived from the files of the Laboratory of Anatomopathology of University Erasmus Hospital, Brussels. Formalin-fixed paraffin-embedded tissues of 45 normal cases as well as 41 benign and 114 malignant tumors were studied. Marked decreases in immunohistochemical galectin-8 expression were observed in colon (p=0.001), pancreas (p=0.007), liver (p=0.0008), skin (p=0.002) and larynx (p=0.02) tissue when comparing malignant tissue to normal tissue and/or benign tumors. The reverse relationship was observed for breast tissue (p=0.007). No statistically significant differences (p>0.05) were detected when comparing normal tissue and/or benign to malignant tumors in lung, bladder, kidney, prostate and stomach tissue. Significant galectin-8 expression was also measured in non-epithelial tissue including tumors of the central and peripheral nervous system as well as in skeletal muscle and mesotheliomas. Immunohistochemical monitoring of galectin-8 thus reveals an organ-type-dependent regulation of expression upon malignant transformation of various tissue types of epithelial origin. This observation will prompt further studies to delineate any relationship with prognosis

Key words: Galectin-8, Immunohistochemistry, Human tissues, lectin, Diagnosis

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Introduction

A growing number of adhesion molecules which play a notable role in tumor cell migration and invasion (Thiery et al., 1988; Streit et al., 1996; Kaltner and Stierstofer, 1998) are referred to as lectins. Currently, these sugar receptors are divided into five families according to their carbohydrate specificities and the structural motif of their carbohydrate recognition domains (CRD) (Gabius, 1997). Of these five groups of lectins, the galectins share a cation-independent binding to galactosides and a jelly-roll motif with a series of invariant amino acids in the binding pocket (Harrison, 1991; Drickamer and Taylor, 1993; Barondes et al., 1994; Kasai and Hirabayashi, 1996; Gabius, 1997, 2000; Hirabayashi, 1997), and display various common features such as a lack of disulfide bridges, glycosylation, and signal sequence and, in most cases, their N-terminal amino acids are acetylated (Kasai and Hirabayashi, 1996; Gabius, 1997; Hirabayashi, 1997). When secreted from cells galectins can interact with a set of extracellular proteins including, for example, laminins, fibronectin and $\alpha_7/\alpha_1\beta_1$ integrins (Harrison, 1991; Drickamer and Taylor, 1993; Barondes et al., 1994; Kasai and Hirabayashi, 1996; Gabius, 1997; Hirabayashi, 1997). Based on the spatial arrangement of the CRD(s), the members of this lectin family are classified into proto-, chimera-, and tandem-repeat types

(Gabius, 1997; Hirabayashi, 1997). In 1994 it was proposed that mammalian galectins be numbered according to the chronology of discovery (registration to GenBank), and on the basis of this system, 11 mammalian galectins have been registered to date (Gabius, 1997; Hirabayashi, 1997; Dunphy et al., 2000). Galectin-1, -2, -5, -7, -10 and -11 belong to the prototype subgroup of galectins; galectin-3 is so far the unique representative of the chimera-type subgroup; and galectins-4, -6, -8 and -9 belong to the tandem-repeat subgroup (Gabius, 1997; Hirabayashi, 1997). Due to the potential of galectins to participate in cell-cell/matrix adhesion, growth regulation and internal processes such as pre-mRNA splicing (Gabius, 1997; Hirabayashi, 1997), it is reasonable to propose an involvement of these proteins in tumor development. Moreover, their interaction with glycans can provide clues on the significance of glycosylation aberrations in malignant transformation, which are currently primarily catalogued but not functionally understood.

To date, only very few data have been published on galectin-8 (Hadari et al., 1995, 1997; Su et al., 1996), prompting to initiate investigation of expression of this endogenous lectin, the first tandem-repeat-type galectin, in tumor histology. cDNA for galectin-8 was cloned by Hadari et al. (1995) from a rat liver cDNA library, and a cDNA encoding human galectin-8 was then isolated from a human brain hippocampus library by means of the full-length rat cDNA used as a probe (Hadari et al., 1997). The product of this clone is a 35-kDa protein consisting of two domains of about 140 amino acids, each of these domains containing a single CRD. These two domains are joined by a 32 amino acid residue-long link peptide (Hadari et al., 1995, 1997), and no homology with any known protein is found in this region (Hadari et al., 1997). Galectin-8 resembles galectin-4 (Oda et al., 1993), galectin-6 (Gitt et al., 1998) and also galectin-9 (Türeci et al., 1997; Wada and Kanwar, 1997) as well as a galectin homologue from *Caenorhabditis elegans* (Hirabayashi et al., 1996), in its typical arrangement of two CRDs covalently linked by a connecting peptide stretch. At nucleic acid level galectin-8 is 50% and 45% homologous with galectin-4 and the nematode galectin, respectively, while at amino acid level galectin-8 shares a 34% and 31% identity (Hadari et al., 1997). Since the two carbohydrate recognition domains of galectin-8 are related but distinct, these two domains are expected firstly to be structurally different and secondly to interact with different sets of carbohydrate ligand, thereby serving as a crosslinking molecule (Hadari et al., 1995; Gabius, 1997).

The aim of the present work is to investigate whether the level of expression of galectin-8 differs significantly between normal, benign and malignant tumors arising from tissues of different embryological origins. For this purpose, we characterized the levels and patterns of immunohistochemical expression of galectin-8 in 200 specimens including 45 normal cases as well as 41 benign and 114 malignant tumors.

Materials and methods

Specimens

A series of 200 retrospective formalin-fixed paraffin-embedded cases were analyzed in order to determine whether they exhibited positivity in immunohistochemical processing aimed at galectin-8 detection. These 200 cases included 45 normal cases as well as 41 benign and 114 malignant tumors. The histological origin of each of the 200 tissues is detailed in the Results. On the one hand, benign, normal or reactively altered tissues, and, on the other, malignant tumors (at least grade II according to the World Health Organization) were studied.

Immunohistochemical staining

Two sections of 5-μm each were taken from each specimen and subjected to processing with the anti-galectin-8 antibody (raised in rabbits against the recombinant protein) and kit reagents. The antibody specificity was ascertained by Western blotting analysis using recombinant galectins-1, -3, -4 and -7 as controls, referring staining with the polyclonal antibody to galectin-8 and its variants with peptide linker extensions. This staining procedure was previously described for the immunohistochemical characterization of various types of galectin-1 and -3 and galectin-binding sites in renal (François et al., 1999), head and neck (Choufani et al., 1999) and smooth muscle (Schwarz et al., 1999) tumors. Briefly, incubation with the anti-galectin-8 antibody was carried out at 25±1 °C for 60 minutes. The dilution used for the anti-galectin-8 antibody was 1:500. Immunoreaction was developed through the application of avidin-biotin-peroxidase complex (ABC) kit reagents (Vector Labs, Burlingame, CA), with diaminobenzidine/H_2O_2 as chromogenic substrates (Choufani et al., 1999; François et al., 1999; Schwarz et al., 1999). The control tissues were incubated with the corresponding pre-immune sera, with the second antibody only, or with the polyclonal anti-galectin-8 antibody preabsorbed with recombinant galectin-8. Omission of the incubation step with the labeled marker served to exclude staining by any of the binding kit reagents, such as the mannose-rich glycoprotein, horseradish peroxidase acid avidin. Counterstaining was carried out with hematoxylin.

Some immunohistochemical patterns of staining (obtained with the anti-galectin-8 antibody in various types of normal tissue, and also in benign and malignant tumors) are illustrated in the Results.

Evaluation of immunohistochemical staining

The staining of the tissue specimens was scored using a semi-quantitative scoring system described by

Bresalier et al. (1997), who characterized the level of the immunohistochemical expression of galectin-3 in human brain tumors. For each specimen analyzed the system proposed by Bresalier and colleagues yields an average staining intensity score that takes into account tumor heterogeneity. All the fields of each single tissue were examined, and the staining intensity and distribution were assessed in each field by means of a x10 objective. The staining intensity was scored as absent (0), weak (1), moderate (2) or strong (3), and the percentage of tumor cells stained was determined. A staining score for each specimen was calculated as "intensity x percentage of positive tumor cells / 100" (Bresalier et al., 1997).

Statistical analyses

The Mann-Whitney rank test (for comparing 2 groups) was used to compare the immunohistochemical galectin-8 expression in normal tissue and/or benign lesions in relation to their malignant counterparts. All the statistical analyses were carried out using Statistica (Statsoft, Tulsa, OK, USA).

Results

Characterization of immunohistochemical galectin-8 expression in normal, benign and malignant epithelia

Figs, 1, 2 detail the staining score values obtained with the anti-galectin-8 antibody in 11 distinct groups of epithelial tissues. The data from these figures clearly indicate that no global conclusion can be drawn with respect to the positivity in these various epithelial tissues. In detail, no statistically significant differences in this parameter were observed between the benign cases (including the normal cases or the benign tumors, or both) and the malignant tumors in the five epithelial tissue groups covering the stomach (Fig. 1), the lung (Fig. 2), the bladder (Fig. 2), the kidney (Fig. 2) and the prostate (Fig. 2).

Breast cancer cases constituted the only group of tissue to exhibit a higher immunohistochemical galectin-8 expression in the malignant as opposed to the benign cases (Fig. 1). This feature is clearly evident in Figs. 3A

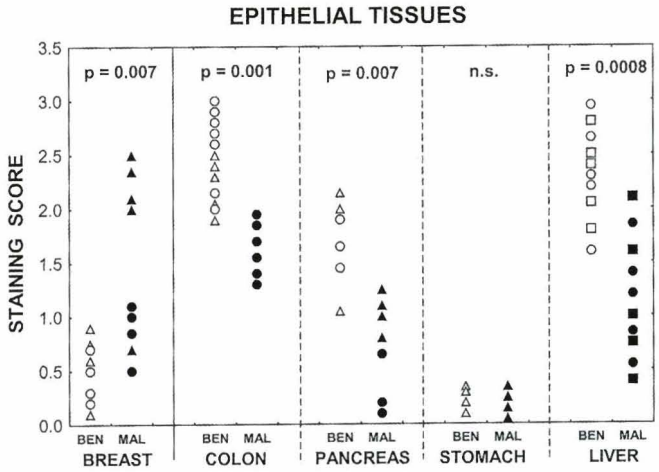

Fig. 1. Characterization of the level of immunohistochemical expression of galectin-8 in 76 specimens including normal or benign (the open symbols in the BEN groups) and malignant tumors (the black symbols in the MAL groups) in epithelial tissue from breast (n=17), colon (n=18), pancreas (n=13), stomach (n=8) and liver (n=20). Immunohistochemical galectin-8 expression was semi-quantitatively determined using a staining score which corresponds to staining intensity multiplied by the % of positive tumor cells / 100. Intensity of staining was scored as absent (0), weak (1), moderate (2) or strong (3). The 17 breast specimens included 4 cases of fibrocystic dysplasia (open dots), 4 tubular adenomas (open triangles), 4 invasive ductal carcinomas NOS (black dots) and 5 lobular carcinomas (black triangles). The 18 colon tissues included 7 cases of normal mucosa (open dots), 5 low to moderate dysplasias (open triangles) and 6 adenocarcinomas (black dots). The 13 specimens referred to as "pancreas" cases included in fact both pancreas and gallbladder cases, e.g. 6 normal (open dots = normal pancreas and open triangles = normal gallbladders) and 7 malignant (black dots = pancreatic adenocarcinomas and black triangles = cholangiocarcinomas) cases. The 8 stomach cases included 4 specimens of normal mucosa (open triangles) and 4 adenocarcinomas (black triangles). The 20 liver cases included 5 normal cases (open dots), 5 cirrhotic livers (open squares), 5 hepatoblastomas (black squares) and 5 hepatocarcinomas (black dots). The statistical intra-group comparisons are listed in the upper part of the figure (n.s.: not significant).

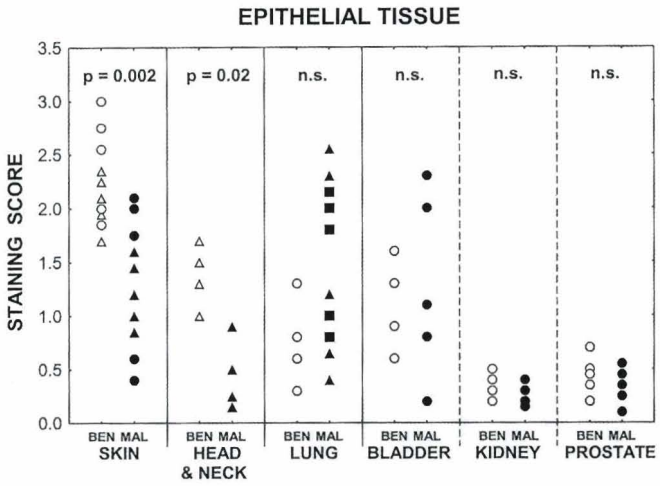

Fig. 2. The legend to Fig. 2 is identical to that for Fig. 1 (except for the origin of the analyzed tumors). Sixty-nine epithelial tissues were analyzed and included skin (n = 20), head and neck (n=8), lung (n=14), bladder (n=9), kidney (n=8) and prostate (n=10) cases. The BEN groups included normal specimens or benign tumors while the MAL groups included malignant tumors. The 20 skin cases included 5 normal specimens (open triangles), 5 *molluscum pendulum* (open dots), 5 spinocellular carcinomas (black triangles) and 5 basal cell carcinomas (black dots). The 8 head and neck cases included 4 normal cases (open triangles) and 4 squamous cell carcinomas (black triangles) of the larynx. The 14 lung specimens included 4 normal cases (open dots), 5 squamous carcinomas (black triangles) and 5 adenocarcinomas (black squares). The bladder group included 4 normal cases (open dots) and 5 transitional carcinomas (black dots). The 8 kidney cases included 4 normal specimens (open dots) and 4 renal carcinomas (black dots). The 10 prostate specimens included 5 benign hyperplasias (open dots) and 5 adenocarcinomas (black dots).

and 3B at morphological level. Higher expression of galectin-8 in the malignant as opposed to the benign breast tissue specimens were mainly detected in lobular carcinomas, one particular subgroup of malignant breast cancers, and not in any type of breast cancer (Fig. 1).

A statistically very significant decrease in immunoreactivity was observed in the remaining 5 groups of epithelial tissues under study when comparing the malignant to the benign tissue. This feature concerns tissue from colon (Fig. 1, 3C,D), pancreas (in fact both pancreas and gallbladder; Fig. 1), liver (Figs. 1, 3E,F), skin and larynx (Fig. 2). In these cases, this decrease was

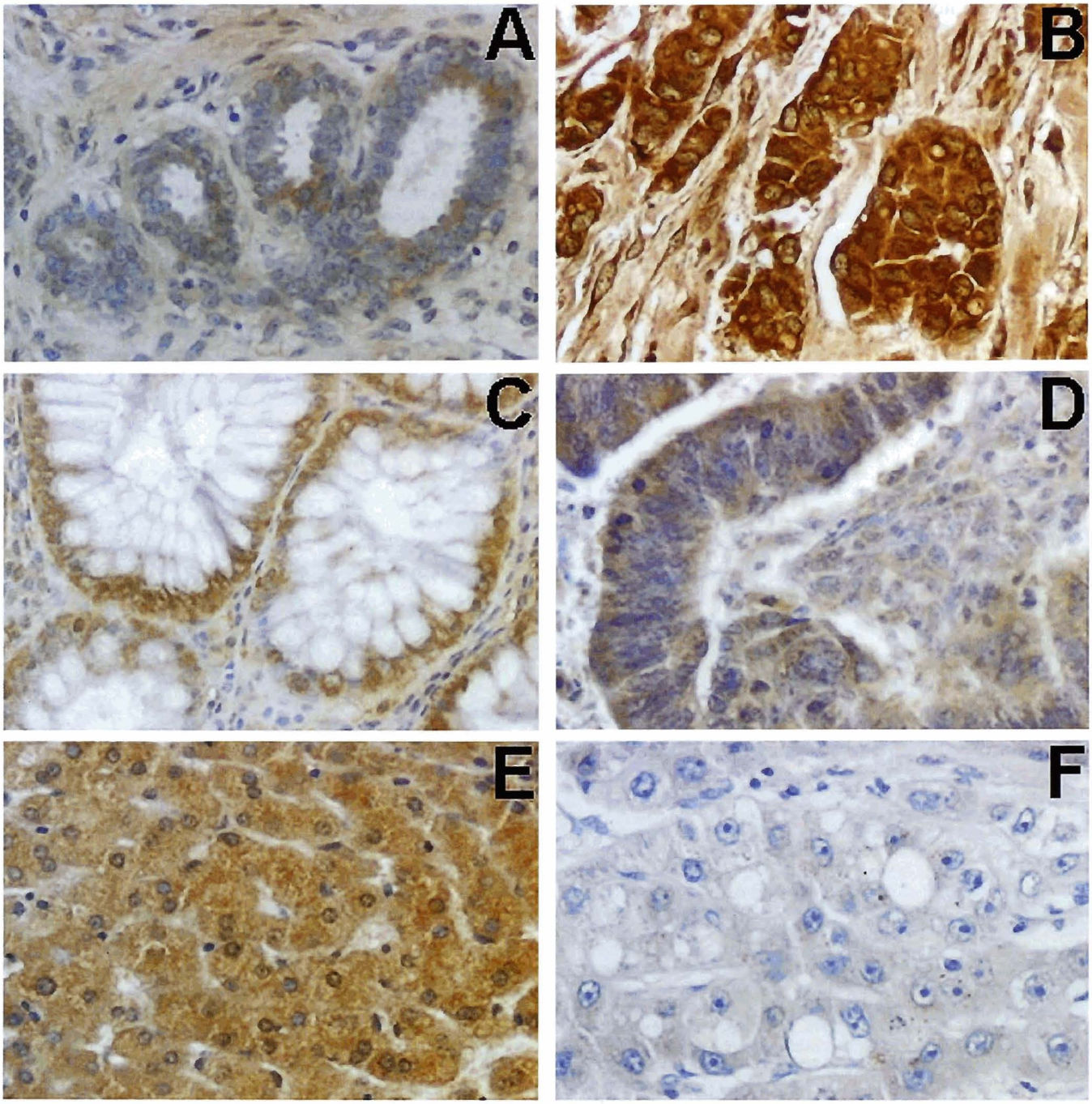

Fig. 3. Morphological illustrations of immunohistochemical galectin-8 expression in sections of a breast tubular adenoma (**a**, x 400), a breast lobular carcinoma (**b**, x 400) normal colonic glands (**c**, x 400), a colon adenocarcinoma (**d**, x 400) a normal liver (**e**, x 400) and a hepatocarcinoma (**f**, x 400). While expression markedly increased from the benign to the malignant breast tissue, it decreased from the benign to the malignant colon and liver tissue.

generally accompanied by a modification in the subcellular location of galectin-8. For example, many nuclei exhibited marked staining in the normal specimens and benign tumors of the colon, while the nuclear location of galectin-8 disappeared in malignant colon.

In order to give some information regarding the distinction between a low percentage of positive cells displaying strong staining intensities and a higher percentage of cells with weak intensities, we detailed (as an example) such data on liver tissues in Fig. 4. In normal and cirrhotic livers (BEN group), the staining intensities of positive cells appeared to be moderate to strong, and the percentage of positive cells per specimens varied between 80 and 100%. In the MAL group (hepatoblastomas and hepatocarcinomas) the percentage of positive cells per specimens varied between 40 and 100%, and the staining intensity of positive cells between weak to moderate.

Characterization of immunopositivity in benign and malignant non-epithelial tissues

Fig. 5 shows that no striking differences were observed between the various groups of tissue of non-epithelial origin with respect to immunohistochemical galectin-8 expression. Indeed, while both the benign and the malignant smooth muscle tumors exhibited low levels of galectin-8, the skeletal tissues, whether normal or malignant, exhibited high amounts. Variable extents of immunopositivity were observed in various groups of tumors of the central and peripheral nervous system (Fig. 5), and also in mesotheliomas (Fig. 5).

Discussion

We herein initiate work on the expression of galectin-8 in various normal or altered tissues and neoplastic lesions. The study involves a large number of tissues with sometimes a low number of specimens in each group. We are aware of this weakness but let us remember that our main goal is to localize tandem-repeat-type galectin in normal specimens, benign and malignant lesions in tissues with different embryological origins. Using Northern analysis Hadari et al. (1995) characterized the expression of galectin-8 in different rat tissues and reported that galectin-8 mRNA is highly expressed in lungs, and, to a lower extent, in liver, kidneys, spleen, hind-limb and cardiac muscles. These

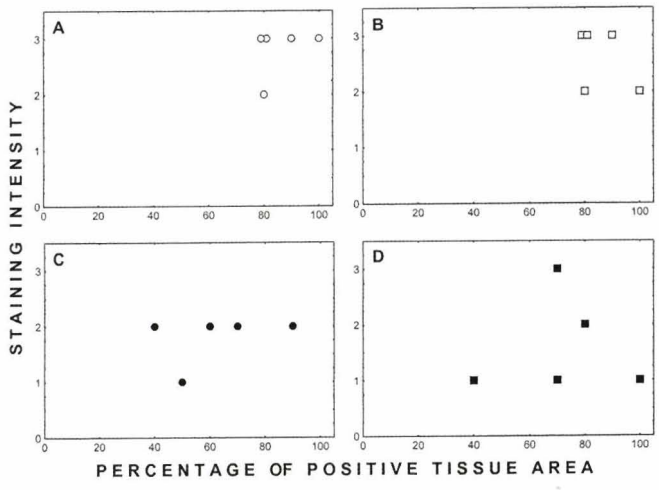

Fig. 4. Expression of galectin-8 in liver tissues based on staining intensity (Y axis) and percentage of tissue stained (X axis). Solid symbols represent individual specimens as follows: normal cases as open dots **(A)**, cirrhotic as open squares **(B)**, hepatoblastomas as black dots **(C)** and hepatocarcinomas as black squares **(D)**.

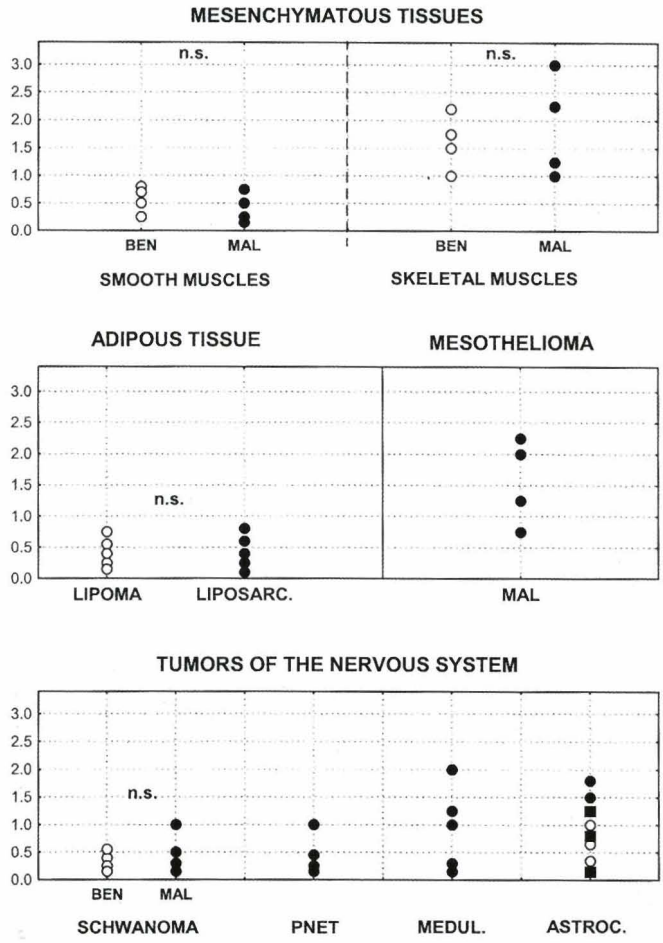

Fig. 5. The legend to Fig. 5 is identical to that for Fig. 1 (except for the origin of the analyzed tumors). Fifty-five cases were analyzed and included 16 mesenchymatous and 10 adipous tissues, 4 mesotheliomas, and 25 tumors of the central and peripheral nervous system. The tissues of mesenchymatous origin included 4 benign (uterine leiomyomas, open dots) and 4 malignant tumors (uterine leiomyosarcomas, black dots), 4 normal skeletal muscles (open dots) and 4 rhabdomyosarcomas (black dots). The adipous tissues included 5 lipomas (open dots) and 5 liposarcomas (black dots). The tumors of the nervous system included 4 benign schwannomas (open dots) and 4 malignant nerve sheath tumors (MNSTs, black dots), 4 primitive neuroectodermal tumors (the PNET group), 5 medulloblastomas (the MEDUL. Group) and 8 astrocytic tumors (3 WHO grade II (astrocytomas, open dots), 2 WHO grade III (anaplastic astrocytomas, black dots) and 3 WHO grade IV (glioblastomas, black squares) cases.

biochemical data on rodent tissues are partly corroborated by our immunohistochemical data on human specimens. Indeed, we also observed significant amounts of galectin-8 in normal lung tissue, but we observed increased levels in normal liver, colon and skin tissue. Hadari et al. (1997) also report that they have been able to demonstrate high levels of galectin-8 expression in 129 human lung carcinoma cells, and argue that like galectin-3, whose expression is also up-regulated in certain cancer types like those of the colon, galectin-8 could represent a promising marker of malignant transformation. However, our data reveal that a high level of galectin-8 is not a general feature of lung cancer. Indeed, we obtained no statistically significant differences for immunopositivity between normal and malignant epithelial lung tissue. In addition, we observed very significant decreases in galectin-8 expression in malignant colon, skin, liver and pancreas tissue, for example, when compared to their normal or benign counterparts.

Following the initial cloning of rat galectin-8 cDNA, surface-epitope masking and expression cloning were instrumental to identify a tumor antigen as a human homologue of galectin-8 (Su et al., 1996). At the protein level, it shares 81% homology with the rat protein. Based on immunological screening it is referred to as human prostate carcinoma tumor antigen (PCTA-1), which is selectively expressed in prostate carcinoma cells but not in normal prostate or benign prostatic hyperplasias (Su et al., 1996). As reported by Hadari et al. (1997), PCTA-1 differs in eight amino acids (at positions from No 56-205) from galectin-8 expressed in human brain, but the reason and consequences for this variability are presently unknown. It is possible that such variability could represent a polymorphism of human galectin-8, for example, or the expression of a mutant form of galectin-8 in prostate carcinoma cells (Hadari et al., 1997). Concerning immunohistochemistry on prostate samples, the RT-PCR data on four cell lines and 15 tissue samples and the immunohistochemical data on frozen sections reported by Su et al. (1996) are not in line with our data on fixed sections. Our data indicate that the cross-reactive protein is expressed at very low levels in normal prostatic tissues as well as in benign hyperplasias or prostatic adenocarcinomas. Still, preliminary results revealed that the level of expression of galectin-8, determined by means of Western blotting, is significantly higher in prostatic adenocarcinomas, compared to normal prostatic tissue, again emphasizing the fact that differences in tissue handling, and the methodologies used for detection, might affect the results.

Conflicting data sets on the levels of expression of galectins-1 and -3 in various organ types were also reported, pointing to the assessment technique, processing, tumor area sampling and selection as non-negligible parameters (Irimura et al., 1991; Lotan et al., 1991; Castronovo et al., 1992; Lotz et al., 1993; Schoeppner et al., 1995). These studies with differences in design and samples intimate that it is essential to be cautious with generalizations. In this respect, it is noteworthy that among breast cancer types immunopositivity was high in the examined lobular carcinomas of the breast, but not in the invasive ductal adenocarcinomas NOS (see Fig. 1).

Concerning potential functional implications, a typical feature of galectins warrants a comment. Galectin-8 lacks the classical signal sequence of a transmembrane region, and despite lacking this sequence, like other galectins (Cooper and Barondes, 1990; Lindstedt et al., 1993; Sato and Hughes, 1994), galectin-8 is externalized by an atypical secretory mechanism. Hence galectin-8, like other galectins, is expected to function, at least in part, extracellularly (Hadari et al., 1997). Atypical secretion is not a unique property of galectins, since other cytoplasmic proteins like thioredoxin (Rubartelli et al., 1992), interleukin-1ß (Siders et al., 1993), basic fibroblast growth factor (Mignatti et al., 1992) and certain S100 proteins (Schäfer and Heizmann, 1996) lack signal sequences, yet are externalized and function extracellularly. The extracellular function of galectin-8 is still unknown but it is intriguing that a PCTA-1-specific monoclonal antibody (11 injections of 200 mg each) reduced the size of DU-145 tumors in seven of nine athymic nude mice (Su et al., 1996). It may well act at several biological levels including cell adhesion, cell proliferation, apoptosis and cell migration, as already evidenced for other galectins (see Gabius, 1997; Kaltner and Stierstorfer, 1998; Perillo et al., 1998; André et al., 1999 for review, and Kopitz et al., 1998 for an example on neuroblastoma cells).

In conclusion, the present data show that immunoreactivity with a galectin-8-specific polyclonal antibody in fixed tissue sections markedly decreases in malignant tissues of the colon, the pancreas, the liver, the larynx and the skin, as compared to their normal or benign tumor counterparts. The reverse relationship was observed for mammary tissue, but a clear explanation cannot be offered presently. These data warrant further investigation that must be carried out on significantly larger series of cases in consideration of inter- and intratumoral heterogeneity to determine whether such decreases in galectin-8 expression are associated with prognosis in these tissues.

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