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ORIGINAL PAPER

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Toward functional glycomics by localization of tissue lectins: immunohistochemical galectin fingerprinting during diethylstilbestrol-induced kidney tumorigenesis in male Syrian hamster

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Abstract The current study focused on galectins (-1, -3, -4, -7, and –8) and deliberately performed immunohistochemical fingerprinting to explore their complexity in a context of experimental renal carcinogenesis. The diethylstilbestrol (DES)-induced renal tumors in male Syrian hamster kidney (SHKT) represent a unique animal model for the study of estrogen-dependent renal malignancies. Kidney sections of DES-treated hamsters (3 days to 11 months of DES exposure) were analyzed by immunohistochemistry using a panel of non-crossreactive antibodies raised against galectins-1, -3, -4, -7, and -8. Levels of expression were quantitatively determined by using computer-assisted microscopy on immunostained tissue sections. Except for galectin-4, all above mentioned galectins were expressed in kidney tumors. Small clusters of galectin-1-positive, most likely preneoplastic cells at the corticomedullary junction were already evident 1 week after DES administration. Galectin-1 and -3 expression was apparently associated with the first steps of the neoplastic transformation, because small tumorous buds were found to be positive after 1 month of treatment. In contrast, galectins-7 and -8 were detected in large tumors and medium-sized tumors, respectively, thereby indicating an involvement in later stages of DES-induced SHKT. Galectins-1, -3, -7, and -8 were also detected by immunofluorescence staining in the HKT-1097 cell line established from SHKT, thus illustrating the stability of galectin expression in tumor cells. Our data document the presence and differential regulation of galectins in the course of renal tumorigenesis in the model of DES-induced SHKT.

Keywords Diethylstilbestrol · Galectin · Glycomics · HKT-1097 · Renal carcinogenesis

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Introduction

Diethylstilbestrol (DES)-induced renal tumors in male Syrian hamster kidney (SHKT) represent a unique animal model for the study of estrogen-dependent renal malignancies. These estrogen-induced neoplasms present an important cytological pleomorphism, making their origin controversial. SHKT were initially described as renal adenomas (Kirkman and Bacon 1950) or adenocarcinomas (Horning and Whittick 1954). Later, Goldfarb and Pugh (1990) disclosed an important histological similarity between early neoplastic buds and DES-induced tubular dysplasia, and proposed to classify SHKT as carcinomas originating from proximal tubules. Recent immunohistochemical investigations have postulated other cells of

origin such as smooth muscle cells (Hacker et al. 1988), a subpopulation of mesenchymal stem cells (Gonzalez and Oberley 1989), juxtaglomerular cells (Dodge et al. 1988), undifferentiated renal interstitial cells (Llombart-Bosch and Peydro 1975; Oberley et al. 1991; Bhat et al. 1993; Cortes-Vizcaino et al. 1994), or dormant germinal cells ectopically located in the kidney (Li et al. 2001). Our group reported the presence of a variety of neuroectodermal markers in SHKT cells both in vitro (Laurent et al. 1999) and in vivo (Toubeau et al. 2001; Nonclercq et al. 2002). Double-label immunofluorescence for S100 and neurofilament revealed that early tumorous buds could stem from S100-positive cells associated with nerve bundles. These observations suggest that DES-induced SHKT originate from a yet unidentified precursor cell present in the sheath of peripheral nerves (Toubeau et al. 2001).

Besides answering the question of the cellular origin, an important issue to be addressed in this tumor model is the definition of biochemical effectors relevant to aspects of the malignant phenotype. An emerging concept in this area is to consider glycan epitopes of cellular glycoconjugates as information-bearing determinants, symbolized by newly coined terms such as sugar code or functional glycomics (Gabius et al. 2002, 2004). Of note, glycosylation is widely known to be prone to malignancy-associated alterations, but this phenomenon is still largely viewed as the result of phenotypic variation and considered at best of diagnostic value (Caselitz 1987; Gabius 1988, 1989, 1997a; Brockausen et al. 1998). Regarding mammalian kidney development and maturation, the identification of distinct spatial and temporal changes in the glycomic profile determined by plant lectins questions the validity of this common assertion (Laitiner et al. 1987; Holthofer 1988). Equally intriguing, inhibitors of glycosylation such as tunicamycin were shown to strongly interfere with nephrogenesis (Ekblom et al. 1979). These lines of evidence have prompted investigators to shift from a phenomenological consideration of glycan display to a more functional perspective assigning to cell surface glycans, especially the accessible branch ends, roles in cell adhesion or growth regulation (Reuter and Gabius 1999; Gabius 2000). As links between glycans and intracellular signaling, the mammalian equivalents of plant lectins used as tools in histochemistry could act as physiological effectors in situ (Villalobo and Gabius 1998; Rüdiger and Gabius 2001). Fittingly, endogenous lectins (i.e., galectins) targeting glycans have emerged during evolution, deciphering code words and translating glycan message into cellular responses (Gabius 1997b, 2001; Rüdiger et al. 2000; Brewer et al. 2002; Rabinovich et al. 2002). With the potential to account for a new class of growth regulators and cell adhesion molecules, their detection and monitoring is warranted, and the following case study encourages further work.

Galectin-3, the only chimera-type family member with unique properties for aggregate formation (André et al. 2001, 2003; Ahmad et al. 2004), has been detected in hamster kidney undergoing developmental regulation (Foddy et al. 1990). The currently suggested spectrum of galectin-3 activities encompasses involvement in growth and differentiation of cells of the mesonephric duct/ureteric bud lineage with terminal differentiation of intercalated cells occurring in interplay with hensin, and in ischemia/reperfusion renal failure (Winyard et al. 1997; Hikita et al. 2000; Nishiyama et al. 2000; Bullock et al. 2001). Interestingly, galectin expression in the kidney is not restricted to this galectin family member, since the presence of proto-type galectin-1 has been documented in human tubular epithelial cells and renal cell carcinomas (Burger et al. 1996; Francois et al. 1999). This lectin reacts very sensitively to modifications of *N*-glycan structure and presentation of glycans in clusters (André et al. 2004a, b). The overlapping ligand profiles of galectins-1 and -3 for glycoproteins of the extracellular matrix and cell surface such as laminin, beta1-integrin, and ganglioside GM1 will not necessarily translate into functional similarity, and divergence has been documented in growth regulation of neuroblastoma cells (Kaltner and Stierstirfer 1998; André et al. 1999; Kopitz et al. 2001). Case studies of galectin-1 have revealed the intimate structural reactivity allowing remarkable ligand recognition (He et al. 2003; Siebert et al. 2003). In the current study, the availability of non-crossreactive antibodies for these two lectins, which maintain target specificity among mammalian galectins (Kaltner et al. 2002), has allowed for the first time a comparative monitoring of their expression profiles. Owing to intracellular functions of these two galectins, for example in pre-mRNA splicing or microdomain association of oncogenic H-Ras (Liu et al. 2002; Rotblat et al. 2004), their intracellular localization, either nuclear or cytoplasmic, was also closely examined.

When looking at the interfamily diversity of galectins (Cooper 2002; Lahm et al. 2004), it is a challenge to move beyond these two family members, especially due to the potential of divergent functionality as pointed out above. Yet, pilot studies establishing immunohistochemical galectin fingerprinting in tumor pathology have underscored the arising importance of this approach (Camby et al. 2001; Nagy et al. 2002, 2003). Using a panel of non-crossreactive antibodies, we were thus able to monitor the presence of proto-type, chimera-type, and tandem-repeat-type galectins in a model of estrogen-induced renal carcinogenesis, and to address the issue of a possible correlation between differential expression and tumor progression.

Materials and methods

Animals and treatment

Male Syrian hamsters (*Mesocricetus auratus*) weighing 70–80 g (3–4 months old) were used throughout the study. The animals were bred and maintained in our animal facility accredited by the Belgian Ministry of Middle Class and Agriculture. The experiments were performed in compliance with the guidelines specified by this institution.

Table 1 Polyclonal antibodies used for the immunohistochemical characterization of diethylstilbestrol (DES)-induced renal tumors by galectin fingerprinting

Antibody	Origin	Dilution
Anti-galectin-1	Rabbit, polyclonal, Pr Gabius	1:100
Anti-galectin-3	Rabbit, polyclonal, Pr Gabius	1:200
Anti-galectin-4	Rabbit, polyclonal, Pr Gabius	1:75
Anti-galectin-7	Rabbit, polyclonal, Pr Gabius	1:50
Anti-galectin-8	Rabbit, polyclonal, Pr Gabius	1:50

Eleven groups of at least four animals were chronically treated with DES following a protocol similar to that previously reported in the literature for the same model (Nogueira et al. 1993). The implants filled with 25 mg DES (Sigma, St Louis, MO) were inserted subcutaneously in the shoulder area of anesthetized animals and renewed every 2.5 months to maintain a constant blood level of DES. Hamsters were killed after 3, 8, and 15 days, and 1, 2, 4, 6, 7, 9, 10, and 11 months of DES exposure. A group of untreated animals (n=8) of various ages (3–9 months) were included as controls.

Immunohistochemical procedures

Immediately after killing, kidney samples were fixed by immersion in Duboscq-Brazil fluid for at least 48 h and embedded in paraffin according to standard procedures. Sections of 4–5 μm thickness were cut serially with a Reichert Autocut 2040 microtome and mounted on silane-coated glass slides. DES-induced renal tumors were then subjected to an optimized protocol to determine the presence of galectins using a panel of antibodies, as applied previously in tumor monitoring (Table 1). As detailed elsewhere (André et al. 1999; Camby et al. 2001; Nagy et al. 2002, 2003), the different galectins were purified by affinity chromatography on lactosylated Sepharose 4B obtained after divinyl sulfone activation (Gabius 1990) and were subjected to rigorous purity and activity controls by one- and two-dimensional gel electrophoresis, nano-electrospray ionization mass spectrometry, gel filtration, ultracentrifugation, hemagglutination, and solid-phase binding assays (André et al. 1999, 2001; Kopitz et al. 2003; Vrasidas et al. 2003; Morris et al. 2004). For each galectin a polyclonal antibody fraction was raised in rabbits and systematically tested by ELISA and western blotting against galectins of the three subfamilies. When crossreactivity was detected, it was removed by performing two cycles of affinity depletion by chromatography on resin-immobilized galectins attached to Sepharose 4B after the activation of the matrix by divinyl sulfone (detailed in Gabius et al. 1991). The specificity of the flow-through IgG fraction was tested again by ELISA and western blot analysis in order to confirm the suppression of crossreactivity.

Tissue sections were immunostained following a slightly modified version of the streptavidin-biotin immunoperoxidase method (ABC method). The sensitivity of the method was increased by microwave pretreatment of dewaxed sections in 0.01 M citrate buffer (pH 6.0) for 2×5 min at a power of 900 W. After microwave treatment, the sections were incubated in 0.4% hydrogen peroxide for 5 min to block endogenous peroxidase activity, and thoroughly rinsed in phosphate-buffered saline (PBS; 0.04 M Na_2HPO_4, 0.01 M KH_2PO_4, and 0.12 M NaCl, pH 7.4). Thereafter, the sections were successively exposed to avidin (0.1 mg/ml in PBS) and to biotin (0.1 mg/ml in PBS) for 20 min to block the reactivity of endogenous biotin. After rinsing in PBS, the sections were incubated in 0.5% casein in PBS for 20 min and exposed sequentially at room temperature to the reagent solutions: (1) primary antisera refined by affinity chromatography over immobilized protein A to obtain IgG fractions at optimal dilutions (see Table 1) for 1 h; (2) biotinylated goat anti-rabbit IgG (diluted 1:50) for 30 min; and (3) ABC complexes for 30 min. Specifically bound peroxidase activity was visualized by incubation with 0.02% 3,3′-diaminobenzidine-0.01% H_2O_2 in PBS. After each step of the immunostaining procedure the sections were rinsed in PBS to completely remove reagents. The sections were finally counterstained with PAS, hemalun, and Luxol fast blue and mounted in a permanent medium. Controls for the specificity of immunolabeling included the omission of the primary antibody or the substitution of non-immune sera instead of the primary antibodies to assess antigen-independent staining. In each case these controls were negative.

The simultaneous detection of galectin-1 and neurofilament proteins by double immunostaining was performed on serial tissue sections obtained from animals killed after 1, 2, 4, or 6 months of DES exposure. The slides were pretreated as described above for the ABC method and then incubated for 1 h at room temperature with a mouse monoclonal anti-neurofilament protein antibody (1:40) mixed with the rabbit anti-galectin-1 antibody to obtain the final dilutions indicated in Table 1. Sections were incubated for 30 min at room temperature with a FITC-labeled goat anti-mouse IgG antibody at a final dilution of 1:50. Thereafter, slides were incubated for 30 min at room temperature with biotinylated swine anti-rabbit IgG antibody. Finally, the slides were incubated for 30 min at room temperature with Texas red-conjugated streptavidin at 1:50 dilution. The labeled sections were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The sections were visually inspected with a Leitz Orthoplan fluorescence microscope equipped with a Ploem system for epi-illumination. Pictures were obtained by a PC-driven digital camera (Leica DC 300F; Leica Microsystems, Heerbrugg, Switzerland). The computer software (KS 400 imaging system; Zeiss Vision, Hallbergmoos, Germany) allowed superimposition of fluorescent images, detection of colocalization, and morphometric analysis.

Grading of DES-induced renal tumors

Neoplasms were graded according to size and gross morphology. More specifically, three stages were defined on the basis of the following criteria: (1) tumorous buds: well-defined, small clusters of tumor cells surrounded by normal kidney tissue (5–200 cells covering a section area of 350 μm^2 to approximately 15,000 μm^2); (2) medium-sized tumors: clusters of increased size with tumor cells infiltrating normal kidney tissue (area >15,000 μm^2 up to 300,000 μm^2; approximate diameter of 150–600 μm); and (3) large tumors: invasive tumors of large diameter extensively infiltrating the kidney (>300,000 μm^2 and up to 80 mm^2). The morphological appearance of the three stages is illustrated in Figs. 1 and 2. In the present work, a total of 224 neoplasms were recovered and analyzed (157 tumorous buds, 15 medium-sized tumors, and 52 large tumors in 70 animals).

Computer-assisted morphometry

The immunostainings were quantitatively characterized by using the software KS 400 imaging system (Zeiss Vision). In this study, the labeling index (LI) refers to the percentage of neoplastic tissue area specifically stained by a given antibody while the mean optical density (MOD) corresponds to the mean staining intensity obtained in immunopositive malignant cells. This method of computer-assisted quantification has been standardized and was described extensively in previous publications (Camby et al. 2001; Nagy et al. 2002, 2003).

Cell culture and immunofluorescence staining

The HKT-1097 cell line (DSMZ number ACC 445) was derived from kidney tumors induced in male Syrian golden hamsters by protracted exposure to DES for a period of 11 months, as described in a previous publication (Laurent et al. 1999). Cell culture was performed at 37°C in a cell incubator with humidified atmosphere at 5% CO_2. For routine propagation, cells were grown in 75-cm^2 flasks containing Dulbecco's modified Eagle's medium (DMEM;

BioWhittaker Europe, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; HyClone, Utah), 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Life Technologies, Merelbeke, Belgium). Cells were passaged once a week, with a renewal of the culture medium every 2 days after seeding. For subculture, the cell monolayers were rinsed with Dulbecco's PBS (DPBS) and dislodged from the vessel bottom by treatment with trypsin/EDTA solution. Concentrations of cells in suspension were measured in a model Z1 electronic cell counter (Beckman Coulter, Fullerton, CA).

For immunofluorescence studies, HKT-1097 cells were plated at a density of 5,000 cells/cm^2 on sterile round glass coverslips in 12-well dishes. Two days after seeding, the cell monolayers were rinsed with DPBS and fixed for 15 min with ice-cold 4% paraformaldehyde (PAF) in the same buffer. After fixation, PAF was changed for DPBS where the cells were kept at 4°C until immunostaining which was performed within the next 20 h.

Detection of galectins by immunofluorescence was performed according to a protocol detailed previously (Nonclercq et al. 2004). In brief, cells monolayers were rinsed several times with PBS (0.04 M Na_2HPO_4, 0.01 M KH_2PO_4, 0.12 M NaCl, 0.1% Triton X-100, pH 7.2). Before exposure to primary antibodies, cells were preincubated for 20 min in PBS containing 5% normal goat serum (PBS-NGS) and 0.05 M NH_4Cl to prevent non-specific adsorption of immunoglobulins. Immunofluorescence detection of galectins-1, -3, -7, and -8 was performed by utilizing as primary reagents rabbit polyclonal antibodies raised against the purified proteins (see above). Cells were exposed for 60 min to one of the primary antibodies diluted 1:50 in PBS-NGS. Thereafter, the cell preparations were incubated for 30 min in the presence of a dextran polymer conjugated with both peroxidase and antibodies raised against rabbit immunoglobulins (EnVision; Dakopatts, Glostrup, Denmark). The next step consisted of a 30-min incubation with rabbit anti-peroxidase antiserum (Laboratory of Hormonology, Marloie, Belgium), followed by exposure to biotinylated swine anti-rabbit immunoglobulins (Dakopatts) for a further 30-min period. Immunolabeling was completed by exposing cells for 30 min to Texas Red-conjugated streptavidin (Vector Laboratories, Burlingame, CA). After final rinses in PBS, the coverslips were mounted on glass slides using commercial anti-fading medium (Vectashield; Vector Laboratories). Negative controls were run by omitting the incubation with the primary antibody. This modification resulted in a virtual disappearance of the signal.

The cell preparations were analyzed by fluorescence microscopy as indicated above. Excitation wavelength of 596 nm and emission wavelength of 615 nm were used for the observation of Texas Red fluorescence. The appearance of immunostained cell preparations was documented by using a PC-driven digital camera (Leica DC 300F; Leica Microsystems). Microscopic fields were digitalized by a software specifically designed for image acquisition and storage (Leica IM 50). Image adjustment and printing were achieved with appropriate softwares (Corel PHOTO-PAINT and CorelDRAW; Corel, Ottawa, ON, Canada).

Western blotting

Kidneys from untreated male hamsters were homogenized in 2.5 vol TRIS-sucrose buffer (25 mM TRIS-HCl, pH 7.4, containing 250 mM sucrose) containing 5 mM EDTA and protease inhibitors. Tissue homogenates were centrifuged at 700 *g* for 10 min, and NaCl and $MgSO_4$ were added to the supernatants to reach a final concentration of 100 and 1 mM, respectively. These supernatants were spun at 100,000 *g* for 1 h. Homogenization and centrifugation were carried out at 4°C. The supernatants of the latter centrifugation were used as cytosolic fractions and assayed for protein content. For western blot analysis, 20 µg cytosolic proteins were resolved by SDS-PAGE on 12% T acrylamide-bisacrylamide gels (15% T for the immunodetection of S100). After separation, the proteins were electrotransferred from the gel onto a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech Europe, Freiburg, Germany). Non-specific binding sites on the membranes were blocked for 3 h at room temperature using a blocking buffer (blotto A) [TBS buffer (10 mM TRIS-HCl, pH 8, 150 mM NaCl) containing 5% non-fat milk and 0.05% Tween 20]. Membranes were then incubated overnight at 4°C with the primary antibody diluted in blotto A. The primary antibodies (anti-galectins-1, -3, -7, -8) used for immunoblotting were the same as those used for immunohistochemical staining. Exposure to the primary antibody was followed by a 2-h incubation at room temperature with peroxidase-conjugated antibodies raised against mouse or rabbit immunoglobulins. Finally, after a 1-min incubation in the presence of BM chemiluminescence blotting substrate (POD), immunoreactive bands were visualized by exposure to a sensitive film (Hyperfilm ECL; Amersham Pharmacia Biotech Europe). Biotinylated molecular weight markers were run in parallel for internal calibration.

Results

The patterns of immunostaining obtained by application of anti-galectin-1, -3, -4, -7, and -8 antibodies in normal renal tissue are described in Table 2. Beside immunohistochemical staining, SDS-PAGE and immunoblotting were performed in order to confirm the expression of chimera- and tandem-repeat-type galectins revealing characteristic bands for galectins-1, -3, and -4 in kidney tissue from untreated animals (data not shown). Galectins-7 and -8 were not detected in kidneys from untreated animals, in accordance with the fact that these proteins are only expressed in a few cells interspersed in several renal compartments (columnar epithelium of the papilla (calyx), cortical interstitial cell, outer medullary interstitial cell, or distal convoluted tubules; Table 2) and representing a minor population as compared to the bulk of renal parenchyma. On the other hand, galectins-1, -3, and -4 which are produced in segments of renal tubules (Table 2) were detectable in control kidneys.

In this study, the development of kidney tumors induced by DES administration to experimental animals followed a course similar to that reported in previous studies (Wattiez et al. 1996; Nonclercq et al. 1998; Toubeau et al. 2001). The first signs of neoplastic transformation, evidenced by the expression of S100 protein, were observed after 4–5 months of exposure to DES,

Table 2 Immunostaining patterns with the different antisera in hamster renal tissue. Abbreviations referring to the different structures and histological types in the kidney are according to a standard nomenclature for renal structures (Kriz and Bankir 1988). (*PTC* Proximal convoluted tubules, *DCT* distal convoluted tubules, *DST* distal straight tubules, *CCD* cortical collecting ducts, *OMCD* outer medullary collecting ducts. Excretory passages: *CEP* columnar epithelium of the papilla (calyx), *TEP* transitional epithelium of renal pelvis and ureter. Renal interstitium: *CIC* cortical interstitial cells, *OMIC* outer medullary interstitial cells, *IMIC* inner medullary interstitial cells)

Antibody (anti-)	Distribution of immunoreactivity
Galectin-1	CCD, OMIC, IMIC
Galectin-3	CCD, OMCD, OMIC, IMIC, TEP
Galectin-4	PTC, DST, CEP
Galectin-7	DCT, CEP
Galectin-8	CIC, OMIC, CEP

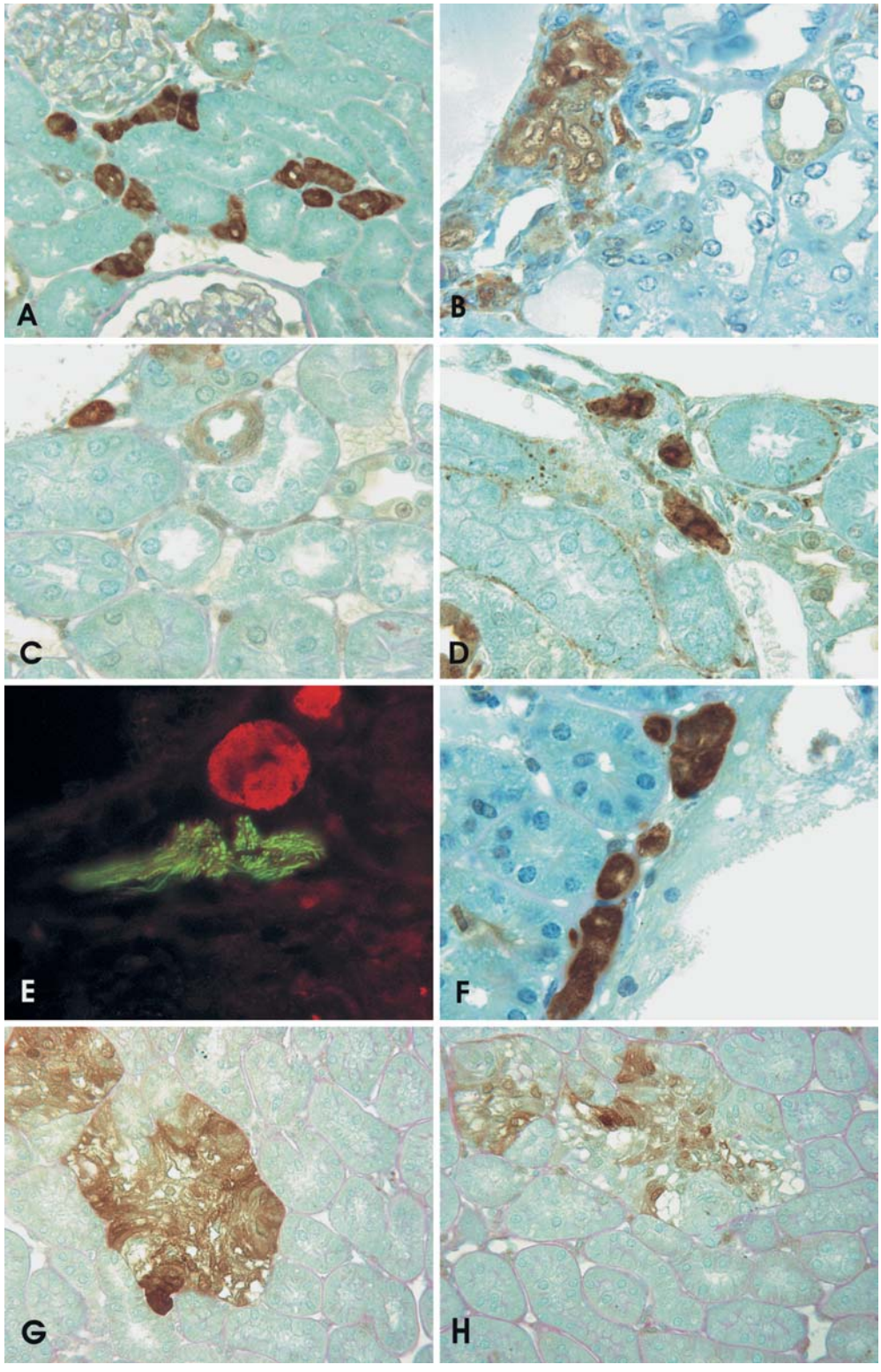

Fig. 1A–H Immunohistochemical localization of galectin-1 (**A**, **C**, **E**, **F**) and galectin-3 (**B**, **D**) in diethylstilbestrol (DES)-induced renal tumors. Experimental animals were exposed to DES for 1 (**A**, **C–E**), 4 (**B**), or 6 (**F**) months. Galectin-1 (**A**) and galectin-3 (**B**) immunoreactivity in neoplastic buds localized at the corticomedullary junction, galectin-1 (**C**) and galectin-3 (**D**) immunoreactivity in isolated cells at the corticomedullary region. **E** Double immunostaining for neurofilaments (FITC, green fluorescence) and galectin-1 (Texas Red, red fluorescence). A galectin-1-positive bud appears adjacent to a small nerve showing neurofilament positivity. **F** Tumorous bud positive for galectin-1 in the subcapsular zone. **G**, **H** Serial sections obtained from medium-sized tumors revealed that cells strongly expressing the S100 protein (**G**) were also weakly positive for galectin-1 (**H**). Magnifications **A** ×320; **B–H** ×650

while most animals killed at 6 months and all animals terminated at 7 months exhibited more advanced tumorous buds. These buds were essentially located at the corticomedullary junction in the vicinity of large blood vessels. Medium-sized tumors were only observed after 7 months while large tumors were recorded in animals treated for at least 9 months. These observations were thus extended by galectin immunohistochemistry which allowed the early detection of putative preneoplastic lesions (evidenced by positivity for galectin-1, see below) 1 week after the DES administration.

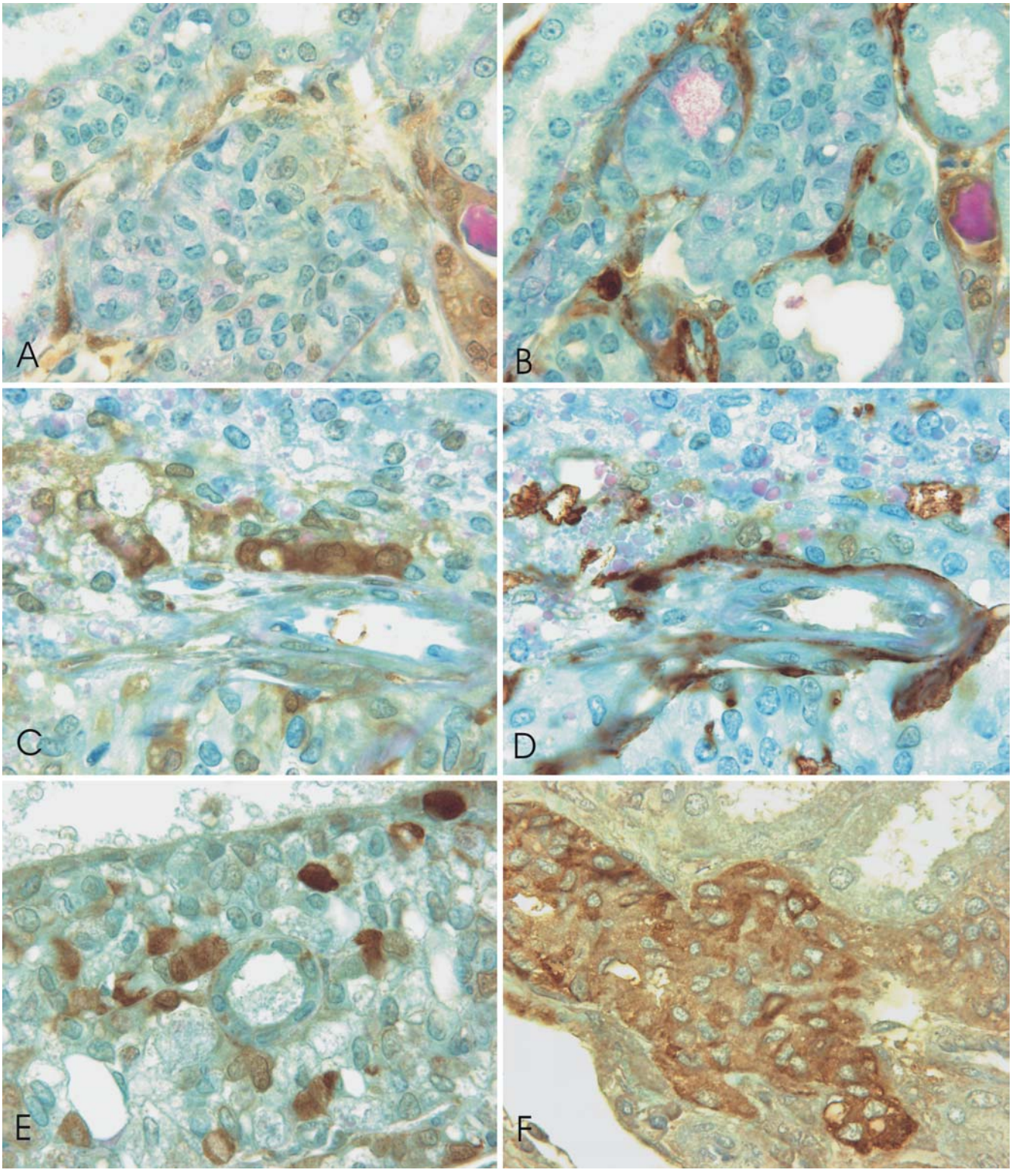

Fig. 2A–F Immunohistochemical localization of galectins in DES-induced renal tumors. Experimental animals were exposed to DES for 7 (**F**), 9 (**A**, **B**) or 11 (**C–E**) months. **A**, **B** Medium-sized tumors negative for galectin-1 (**A**) and galectin-3 (**B**). Positive interstitial cells display a typical fusiform appearance. **C**, **D** Serial sections of a large tumor. Small clusters of cells are positive for galectin-1 (**C**), whereas scattered cells express galectin-3 (**D**). **E** Galectin-7-positive cells scattered in a large tumor. **F** Medium-sized tumor positive for galectin-8 localized at the corticomedullary junction. Magnifications **A–F** ×650

Table 3 Galectin immunostaining of renal tumors in male Syrian hamster kidney. [*MOD* Mean optical density (values ranging from 0 to 255), *LI* labeling index (in percent)]

		Duration of DES exposure (months)								
		0.5	1	2	4	6	7	9	10	11
Galectin-1										
Small tumor	MOD	213	250	202	223	223	223	226		213
	LI	63	73	77	71	80	70	65		59
Medium tumor	MOD					23	0	150		
	LI					<1	0	<1		
Large tumor	MOD								59	125
	LI								<1	6
Galectin-3										
Small tumor	MOD		223	219	244	237	229	241		
	LI		84	84	76	82	54	68		
Medium tumor	MOD						0	0		
	LI						0	0		
Large tumor	MOD							195	223	225
	LI							0.5	2.4	3.5
Galectin-7										
Small tumor	MOD									
	LI									
Medium tumor	MOD									
	LI									
Large tumor	MOD								168	181
	LI								0.9	0.7
Galectin-8										
Small tumor	MOD									
	LI									
Medium tumor	MOD						168			
	LI						43			
Large tumor	MOD									
	LI									

The immunolabeling of neoplastic tissue with the different anti-galectin antibodies is illustrated in Figs. 1 and 2. The results obtained from the morphometric analysis of positive cell populations are presented in Table 3. Galectins-1 and -3 appeared very early during the first steps of the neoplastic transformation since the first small clusters of positive cells were detected after 1 month following the beginning of DES administration. Within these clusters, antibodies raised against galectins-1 and -3 produced a uniform and intense labeling both in the nucleus and in the cytoplasm of all cells. The morphometric analysis of galectin-positive buds revealed that the MOD remained high regardless of the duration of DES exposure. Similarly, the LI was high for the first 6 months but showed a slight decrease from 7 through 11 months. In more than 90% of our observations, these positive buds were localized at the corticomedullary junction in the perivascular connective tissue, next to large arcuate blood vessels (Fig. 1B). In some sections, occasional positive buds were observed in kidney cortex, generally in the subcapsular region (Fig. 1F). Focally, we have also detected galectin-1-positive buds in the periphery of juxtamedullary glomeruli (Fig. 1A). Finally, as illustrated in Fig. 1C, D, for galectins-1 and -3 immunoreactivity was detected in isolated cells scattered in the interstitium. In most cases, the monitoring of consecutive sections showed that these positive cells were distributed in the vicinity of small positive tumorous buds. Using anti-galectin-1 antibody, we also detected several small clusters of isolated cells at the corticomedullary junction already after 1 week of DES administration. Since these galectin-1-positive clusters were observed rather soon after treatment, we considered them to be preneoplastic clusters. The first galectin-1- and galectin-3-positive tumorous buds were detected rather soon after DES administration but it should be noted that they were also observed later, up to 11 months, next to larger tumors. Galectins-4, -7, and -8 immunoreactivities were not detected in small neoplasms regardless of the duration of DES administration. In the kidneys of control animals, no positive single cells or cell clusters similar to those observed in treated kidneys were ever disclosed by either anti-galectin antibody used in this study.

After 6–7 months of treatment, expression of galectins-1 and -3 concerned only a small number of weakly positive cells in medium-sized tumors, where these cells presented a focal distribution. The observation of serial sections obtained from medium-sized tumors revealed that cells strongly expressing the S100 protein were also weakly positive for galectin-1 (Fig. 1G, H). Immunoreactivities for galectins-1 and -3 were also detected in fusiform cells in the interstitial tissue, mainly around medium-sized neoplasms (Fig. 2A, B). The cytoplasm of a majority of cells in medium-sized tumors also appeared positive for galectin-8 (Fig. 2F) but this marker was not detected in tumorous buds or in large tumors. The medium-sized tumors, irrespective of their extension or of the duration of animal treatment, were always negative when tested with anti-galectins-4 and -7 antibodies.

In large tumors, the expression of galectins-1, -3, and -7 appeared heterogeneous (Fig. 2C–E), with scattered positive cells displaying moderate to intense immunola-

beling. The morphometric analysis confirmed that the LI in these tumors was very low as compared to tumorous buds. However, the MOD remained high for galectin-3 and moderate for galectins-1 and -7. The expression of galectins-1 and -7 was moderate both in nucleus and cytoplasm of positive cells while galectin-3 immunoreactivity only remained conspicuous in the cytoplasm. Cells positive for galectin-3 were generally larger than surrounding neoplastic cells and characterized by oval shape and dense nuclei (Fig. 2D). The observation of serial sections obtained from large tumors demonstrated that cells expressing galectin-3 were mostly distinct from galectin-1-positive cells (Fig. 2C, D). These large tumors were negative for anti-galectin-4 and -8 antibodies.

These observations suggest that galectins-1 and -3 are associated with the very early stages of neoplastic transformation since they could already be detected 8 days after the beginning of DES administration. On the other hand, galectins-7 and -8 are only expressed in large tumors and medium-sized tumors, respectively, and thus appear to be involved in more advanced stages of SHKT development.

In a previous publication (Toubeau et al. 2001), we have suggested that SHKT could originate from a yet unidentified precursor cell present in the sheath of peripheral nerves. In the context of this study of galectin expression in tumorous cells, we have analyzed by double immunofluorescence the relative distribution of nerve fibers, identified by the detection of neurofilaments, and galectin-1-positive tumorous buds. These investigations concentrated on tumorous buds detected in the kidney of hamsters exposed to DES for 1–6 months. In this context, the occurrence of tumor cells associated with nerve bundles was occasional, but we have nevertheless found three fields with the relevant structures. In all cases, the neurofilament-positive nerve fibers were observed in the vicinity of galectin-1-positive buds (Fig. 1E) but we were never able to pinpoint unambiguous colocalization.

The appearance of galectin immunoreactivity during DES-induced renal carcinogenesis suggests that members of this family of proteins could be closely associated with neoplastic transformation and tumor progression in this particular model. It ensues that galectin production could be a phenotypical characteristic of at least a subpopulation of tumor cells. In order to further address this issue and to ascertain the availability of a suitable cell culture model, galectin expression was examined by immunofluorescence staining in the HKT-1097 cell line derived from SHKT. Actually, HKT-1097 cells were found to be positive for the same galectins as those demonstrated in SHKT, i.e., galectins-1, -3, -7, and -8, even though there was a striking variation in the immunofluorescence staining pattern (Fig. 3). Immunostaining for galectins-1 (Fig. 3A) or -8 (Fig. 3F) resulted in cytoplasmic fluorescence, whereas galectin-7-associated immunoreactivity was mostly nuclear (Fig. 3E). Galectin-1 signal was rather uniform in the cytoplasm of positive cells, as compared to galectin-8 immunofluorescence which frequently appeared perinuclear (Fig. 3F). Most variability was seen in the case of galectin-3 expression and intracellular distribution. Thus, cells with a weak intensity of fluorescence were often observed in the vicinity of cells exhibiting a strong signal (Fig. 3B). The immunofluorescence signal in positive cells was nuclear or cytoplasmic (Fig. 3C). In the latter cells, the immunofluorescence pattern suggested an uneven distribution of cytoplasmic immunoreactivity (Fig. 3B, D). Finally, as shown in Fig. 3A, B, D, and F, immunofluorescence staining with anti-galectin-1, -3, and -8 antibodies typically decorated cell processes. These observations not only document the stability of galectin expression in transformed renal cells but also indicate intercellular heterogeneity in the expression of these proteins.

Discussion

The estrogen-induced tumor in male SHK represents a unique model for the study of estrogen-dependent renal malignancies (Li and Li 1984, 1990; Liehr et al. 1986; Liehr 1997, 2001; Li et al. 2001). There is a controversy about the cell of origin and the processes involved in this model during tumorigenesis (Hacker et al. 1988). Different studies, as outlined in the Introduction, have suggested that the cells of origin might be interstitial stem cells mainly found at the corticomedullary junction (Oberley et al. 1991), cells of the juxtaglomerular apparatus (Dodge et al. 1988), vascular smooth muscle cells (Hacker et al. 1988), or unidentified dormant germinal cells ectopically located in kidney (Li et al. 2001). A recent immunohistochemical study raised the possibility that DES-induced SHKT could originate from a yet unidentified precursor cell located in the sheath of peripheral nerves (Toubeau et al. 2001).

Carcinogenesis and differentiation are invariably accompanied by changes in the profile of glycan determinants (glycomics). These modifications are traditionally monitored by plant lectins and interpreted phenomenologically. Because of the growing interest in the role of tissue lectins, our attention has turned to the identification of the versatile roles of glycans (functional glycomics). The spatially accessible branch ends of glycans are ligands for members of the galectin family of endogenous lectins. Galectins are involved in regulating cell proliferation, apoptosis, and migration. The present study has investigated the profile of expression of galectins in the hamster kidney after exposure to DES. We have analyzed the expression of galectins-1, -3, -4, -7, and -8 from the early stages of neoplastic transformation to advanced tumors observed after 11 months of treatment. We have already established that S100, Leu-7, and vimentin were reliable markers of early tumor development (Toubeau et al. 2001). Using the latter markers, the earliest signs of transformation were not observed prior to 4 months of DES exposure. Using an anti-galectin-1 antibody, we have now detected the first small clusters of positive cells, presumably preneoplastic cells, at the corticomedullary junction 1 week after starting DES administration. After

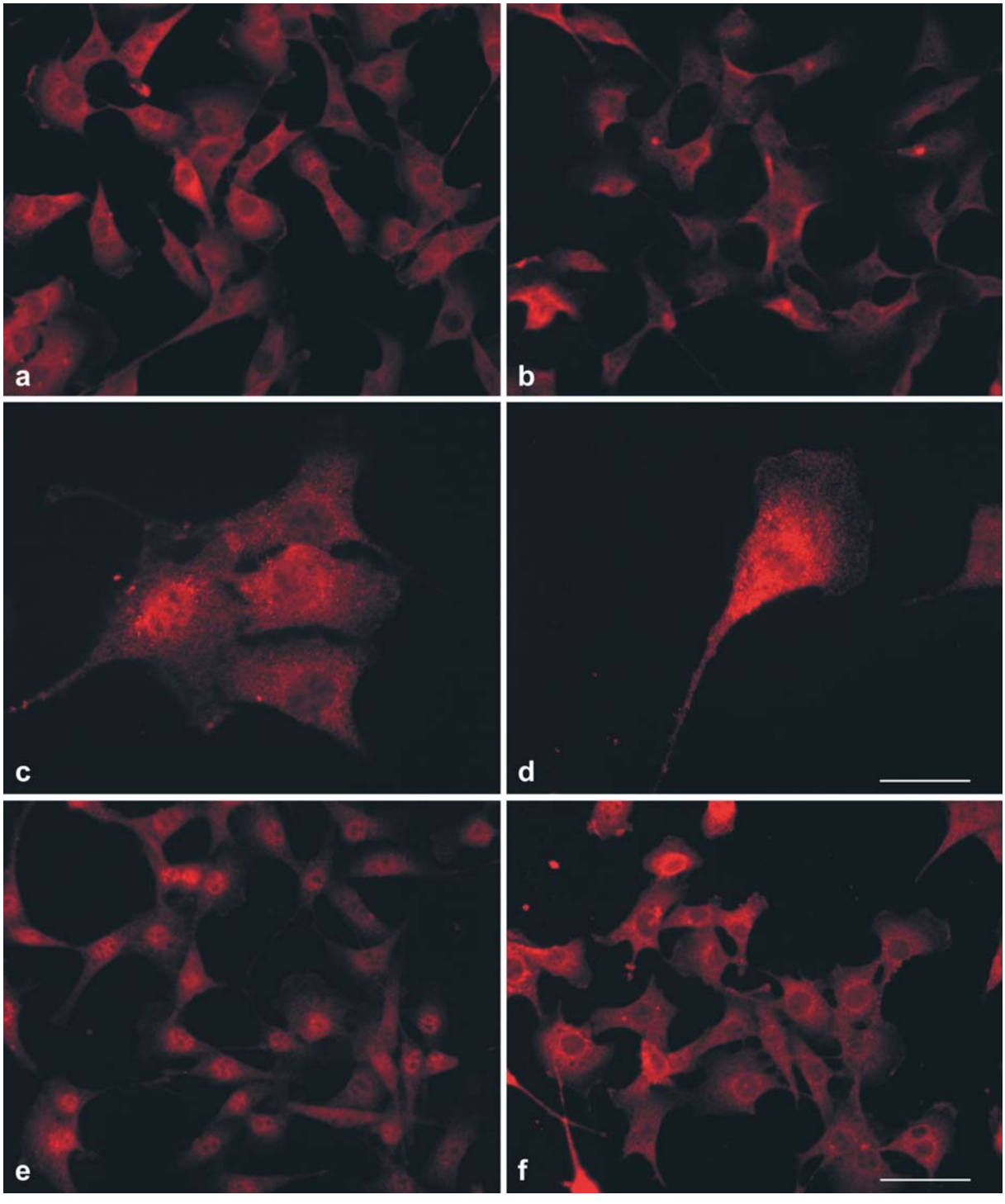

Fig. 3A–F Immunofluorescence detection of galectins in the HKT-1097 cell line. Cells were processed for immunofluorescence staining using primary antibodies raised against galectin-1 (**A**), galectin-3 (**B–D**), galectin-7 (**E**), or galectin-8 (**F**). Texas Red labeling. *Bars* 50 μm in **A**, **B**, **E**, **F**; 25 μm in **C**, **D**

1 month of DES exposure, galectin-1- and galectin-3-positive tumorous buds were observed at the corticomedullary junction in the perivascular connective tissue next to large blood vessels. Our observations suggest that galectin-1 and -3 may be involved in the early stages of DES-induced SHKT. Such an implication of these galectins in the early stages of carcinogenesis was also described in human colon cancer (Nagy et al. 2002). These authors have attributed a significant prognostic value to galectins-1, -3, and -4 in Dukes A and B (early stages) when they are expressed in colon cancers, and galectins-1

and -3 were found in vitro to significantly affect migration of colon cancer cells (Hittelet et al. 2003).

In medium-sized tumors, only a very small number of cells displayed a weak galectin-1 and -3 immunoreactivity. However, galectin-1 and -3 immunolabeling also appeared in fusiform cells of the interstitial tissue surrounding medium-sized tumors. A similar galectin-1 immunoreactivity in peritumorous tissue has also been reported in the stroma surrounding ovary (van den Brule et al. 2003), thyroid (Xu et al. 1995), head and neck (Gillenwater et al. 1996), colon (Sanjuan et al. 1997), and prostate carcinomas (van den Brule et al. 2001). In ovary carcinoma, the use of in situ hybridization showed that fibroblast cells, adjacent to malignant cells, expressed galectin-1 mRNA and contributed to galectin-1 expression around the tumor. It can be assumed that malignant cells contribute, in a paracrine fashion, to this peritumorous galectin-1 expression, since conditioned medium obtained from ovary carcinoma cells induced increased galectin-1 expression in 84 BR fibroblasts. Another possible role for galectin-1 accumulating around the tumor could be the modulation of a local antitumor immune response. Indeed, galectin-1 induces the apoptosis of activated T lymphocytes and could therefore be involved in the barrier protecting cancer cells against the immune response generated by the host (Rabinovich et al. 2002; Rappl et al. 2002).

After 7 months of treatment, the cytoplasm of most cells in medium-sized tumors appeared galectin-8 positive. This observation confirms a previous report about galectin-8 expression in human colon cancer (Nagy et al. 2003), suggesting that galectin-8 expression also presents a prognostic value in intermediate and late clinical stages (Dukes C and D stages).

In large tumors, the expression of galectins-1, -3, and -7 was rather heterogeneous and was characterized by scattered positive cells displaying a moderate to intense immunolabeling. The observation of consecutive sections demonstrated that cells expressing galectin-3 were different from galectin-1-positive cells. These galectin-3-positive cells were characterized by oval shapes and dense nuclei. A recent study confirmed that the overexpression of galectin-3 can exert a protective effect against apoptosis (Matarrese et al. 2000). The anti-apoptotic effect of galectin-3 could result from the ability of this molecule to heterodimerize with bcl-2, a well-known anti-apoptotic effector (Yang et al. 1996). Galectin-7 is specifically expressed in keratinocytes at all stages of epidermal differentiation or in papillary thyroid carcinomas (encapsulated follicular variant) (Magnaldo et al. 1995; Rorive et al. 2002). The galectin-7 gene was also overexpressed in chemically induced mammary carcinomas (Lu et al. 1997). In accordance with these observations, our data also disclosed an upregulation of galectin-7 in large tumors. Galectin-7 expression was restricted to the terminal stages of DES-induced SHKT, since tumorous buds and medium-sized tumors were never positive for this galectin.

The nuclear expression of galectin-3 remained intense in tumorous buds and in large tumors while its cytoplasmic expression tended to decrease (Fig. 2C, D). Different reports have shown downregulation of galectin-3 expression in cytoplasm of ovary, uterus, and breast carcinomas (van den Brule et al. 1994, 1996; Castronovo et al. 1996). On the other hand, other authors have demonstrated that cytoplasmic galectin-3 expression is upregulated in cancers of tongue, thyroid, liver, stomach, central nervous system, and colon (Lotan et al. 1994; Xu et al. 1995; Bresalier et al. 1997; Hsu et al. 1999; Inohara et al. 1999; Honjo et al. 2000). It still remains unclear why the regulation of galectin-3 expression is different in various organs during neoplastic progression. The divergent expression of cytoplasmic and nuclear galectin-3 during neoplastic progression of SHKT suggests different biological roles for galectin-3, reflected by its subcellular localization. In normal epithelium, nuclear and cytoplasmic galectin-3 could be associated with proliferation and differentiation, respectively. Previous studies have already shown that: (1) mitogenic stimulation of quiescent fibroblasts causes a prompt increase of nuclear galectin-3 expression (Moutsatsos et al. 1987; Agrwal et al. 1989); and (2) that nuclear galectin-3, combined with its interacting ligand Gemin 4 (Wang et al. 1995), is involved in pre-mRNA splicing (Dagher et al. 1995). Besides, nucleocytoplasmic translocation of galectin-3 is associated with antiapoptotic activity (Takenaka et al. 2004).

According to different studies, the estrogen-induced renal tumorigenesis in the hamster seems strictly dependent of estrogen exposure (Yager and Liehr 1996; Li et al. 2001; Toubeau et al. 2001). Moreover, the presence of estrogen receptors has been reported in Schwann cells growing in vitro (Jung-Testas et al. 1994; Do Thi et al. 1998) as well as in the HKT-1097 cell line derived from SHKT (Laurent et al. 1999). The addition of DES to an estrogen-free medium stimulated growth in HKT-1097, even though these cells appeared much less responsive than MCF-7 cells which are considered as a paradigm of estrogen-dependent cells (Brohée et al. 2000). In the uterus of ovariectomized mice treated with a single injection of 17 β-estradiol, the level of galectin-1 mRNA already increased 6 h after estrogen administration and remained high up to 24 h postinjection (Choe et al. 1997). The high level of galectin-1 expression in the early stages of SHKT (observed in preneoplastic cells) may therefore derive from an estrogen-dependent, so far unidentified precursor cell located in the sheath of peripheral nerves. Our observations obtained from double immunostaining suggest that there is a close association between galectin-1-positive, small tumorous buds and neurofilament-positive nerve fibers.

We are well aware that the observations reported here still remain largely descriptive. However, they should be taken as an incentive to proceed further and try to explain why estrogens induce neoplastic transformation of this unidentified precursor cell, why galectins-1 and -3 are present in the first stages of neoplastic transformation, and why galectins-7 and -8 appear in later stages. This

would establish and validate an attractive model with temporally distinct regulation of galectin expression.

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