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Lectin Histochemistry as a Predictor of Dysplasia Grade in Colorectal Adenomas

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Lectins are sugar-binding proteins that bind to specific cellular carbohydrates, commonly affecting cellular physiology. Phaseolus vulgaris leucoagglutinin (PHA), ulex europaeus isoagglutinin-I (UEA-I), wheat germ agglutinin (WGA) and peanut agglutinin (PNA) are among the most well studied lectins in various tissues. The purpose of this study was to detect the above lectins' binding sites and so examine alterations in glycoconjugate expression in neoplastic cells of 52 colorectal adenomas with various clinicopathologic characteristics and proliferation rates. Lectin histochemistry was performed in paraffin sections with and without neuraminidase treatment. Proliferative fraction was determined by immunolabelling for Proliferating Cell Nuclear Antigen. PHA was the more frequently positive lectin in the examined specimens; however, it was simultaneously detected in normal colonic mucosa and so was WGA. The frequency of high grade dysplasia was significantly greater in older patients and in samples with UEA-I positivity without neuraminidase pretreatment. UEA-I-reactive adenomas were generally characterized by high cell proliferation rates. A statistical model based on patients' age and UEA-I binding without neuraminidase treatment can generally predict grade of dysplasia in 83% of adenomas and particularly high grade dysplasia in up to 93% of adenomas; so, such a model may be potentially useful for the early detection of neoplasia, for instance in exfoliative cells from the large intestine. (Pathology Oncology Research Vol 6, No 4, 265–271, 2000)

Keywords: colorectal adenomas, lectin binding, phaseolous vulgaris leucoagglutinin, ulex europaeus, wheat germ agglutinin, peanut agglutinin

Introduction

Colorectal carcinogenesis is known to involve multiple steps from hyperproliferative epithelium through adenoma formation to cancerous stages. As concerns sporadic carcinogenesis of the large intestine, no consistent genetic abnormalities have so far been defined. Most cancers of the colon are preceded by an adenomatous polyp (adenoma).[1] The well established risk factors for an adenoma's malignant transformation (i.e. max. diameter >2cm, villous histology and severe dysplasia in particular) form the basis for the prediction of an adenoma's behaviour.

Adenomatous colonic polyps are not totally homogeneous structures; a single polyp may contain areas of varying morphology and contain varying degrees of dysplasia and hence an altered basal mitotic rate.[2] Neoplastic cell transformation is associated with altered cell surface membranes and particularly with an altered carbohydrate composition. Reliable biomarkers that differentiate unequivocally normal, hyperplastic, adenomatous and cancerous states in the large bowel do not exist. In fact, carbohydrate-recognition mechanisms have been implicated in specific cellular interactions that also occur during normal cell growth and differentiation.[3] The above mentioned interactions may be mediated at least in part by carbohydrate-binding proteins (lectins) produced by vertebrate cells.

Lectins are glycoproteins of non-immunologic origin that bind to define simple or complex carbohydrate motifs

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Abbreviations: PHA: Phaseolous vulgaris leucoagglutinin, UEA-I: Ulex europaeus isoagglutinin-I, WGA: Wheat germ agglutinin, PNA: Peanut agglutinin, PBS: Phosphate buffer saline

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acting as lectin receptors. As such, lectins are useful tools to study the glycoprotein and glycolipid cellular structure. Altered glycosylation detected by lectin binding has been found after mitogenic stimulation of quiescent cells, induction of cell differentiation, malignant transformation, tumor progression and acquisition of the metastatic phenotype[3]. Some lectins are mitogenic themselves and have, therefore, been used as growth stimulators in cell culture systems.[4]

The lectin staining properties within individual tissues, may have a relevance to the neoplastic process or may reflect the biological nature of the tumor. Phaseolus vulgaris agglutinin (PHA) is a common dietary lectin, isolated from red kidney beans, which binds to β1,6 branched oligosaccharides[5] and has been associated with poor prognosis in human breast and colon cancers.[4] The lectin of ulex europaeus isoagglutinin-I (UEA-I) combines with the H antigen that corresponds to blood group O and binds mainly to endothelial cells.[6] Wheat germ agglutinin (WGA) is a dietary lectin and binds to colonic receptors.[7] Peanut agglutinin (PNA) increases proliferation of the colonic mucosa[8,9] and has been shown on tissue sections to bind selectively to a mucin glycoprotein, the T antigen, produced by colon cancers. It is theorized that increasing expression of the T-antigen and other abnormal surface antigens on increasingly dysplastic tissues reprises the adenoma to carcinoma sequence.[10] The sugar moieties recognized by the above mentioned lectins are shown in *Table 1*.

For the early detection of colon neoplasia eg. by noninvasive screening tests examining exfoliative cells from the large bowel mucosa, new markers indicative of malignant transformation are needed since mere morphology and proliferative capacity may often be misguiding factors. To be more precise, dysplasia-like alterations in cell morphology and an increase in growth fraction may actually accompany regeneration of colon mucosa, without any underlying malignant potential. Histochemical techniques have been used to demonstrate a variety of alterations in glycoconjugate synthesis by phenotypical changes in lectin binding which reflect functional abnormalities

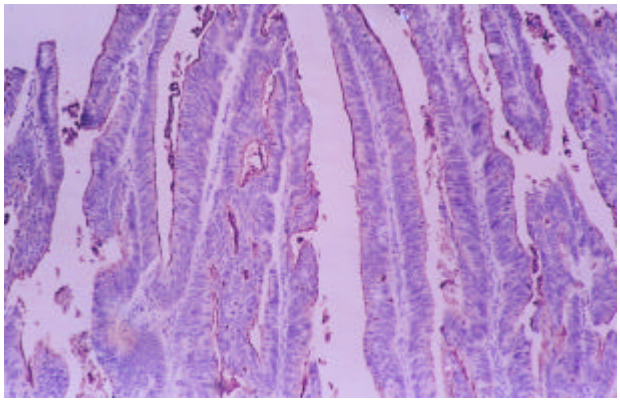

Figure 1. UEA-I binding at the surface of columnar neoplastic cells of a villous adenoma with low grade dysplasia (UEA-I histochemistry, original magnification x200).

potentially related to malignant degeneration. In this study we attempted to study a panel of the above mentioned lectins which bind to different sugar motifs, in relation to dysplasia degree, to two patient features of uncertain clinical significance (i.e. patients' age and sex) as well as to cell proliferation rates in a series of 52 colorectal adenomatous polyps.

Materials and Methods

Fifty-two patients with sporadic adenomas were enrolled in our study [mean age (range): 73 (48-88), male/female ratio: 34/18]. As regards the location of the adenomas, 9 were located at the right colon, 29 at the left colon and 14 at the rectum. Thirty-two samples measured less or equal to 10 mm and the rest had a maximum diameter of more than 10 mm. All tissues were originally fixed in buffered formalin and embedded in paraffin in the routine fashion. Histological diagnosis was reached by evaluation after conventional haematoxylin and eosin staining. Apart from the adenomatous tissue, the majority of sections contained contiguous, histologically normal mucosa. Histologically, the breakdown of the examined adenomatous polyps was as follows: four villous adenomas, thirteen tubulovillous adenomas and thirty-five tubular adenomas. Tumors and moderate cytologic atypias and architectural abnormalities (n=29) were categorized in the group of low-grade (GI) dysplasia while those samples with severe epithelial atypias including those with focal areas of "intraepithelial" and "intramucosal" carcinoma were grouped as high-grade (GII) dysplastic adenomas.

Table 1. **Major sugar binding specificities of lectins used in this study**

Lectin from	*Abbreviation*	*Carbohydrate specificity*	*Inhibitor*
Phaseolus vulgaris	PHA	N-Acetyl-D-Galactosamine	N-Acetyl-D-Galactosamine
Ulex europaeus I	UEA-I	α-L-Fucose	L-Fucose
Triticum vulgaris	WGA	N-Acetyl-D-Glucosamine N-Acetyl-neuraminic acid	N-Acetyl-D-Glucosamine
Arachis hypogaea	PNA	Galactosyl-β-(1-3)-N-Acetyl-D-galactosamine	D-Galactose

Histochemistry was performed with Dako „antibodies“ (Glostrup, Denmark) for all four lectins examined. Unstained tissue sections were heated in an oven at 56°C for 20 minutes and then deparaffinized, brought to water and washed in phosphate buffered saline (PBS) for five minutes. Endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide-methanol solution for 30 minutes at room temperature. Trypsinization was not performed. The slides were then washed twice in PBS for ten minutes each. Each case was studied with and without prior neuraminidase digestion (+N, -N respectively). It is accepted that the enzymatic digestion with neuraminidase exposes cryptic binding sites[11] and unmasks sequences (by cleaving off protein-linked carbohydrates and removing terminal sialic acids) so the binding intensity is considerably increased. Those cases treated with neuraminidase were first treated with brief saponification for ten minutes. Slides were rinsed in PBS for 15 minutes and layered with 100 U/mL of neuraminidase (Calbiochem, San Diego, CA, USA) in a 0.1 mol/L acetate, 0.04 mol/L calcium chloride buffer at pH 5.5 and incubated overnight at 37°C. After overnight digestion with neuraminidase, both neuraminidase and non treated sections were washed in PBS for 15 minutes at room temperature. Sections were incubated for 30 minutes at room temperature with biotin-labelled lectins (PHA, UEA-I, WGA or PNA) that had been diluted in PBS to a concentration of 50 mg/mL. Slides were then washed four times in PBS (five minutes each) and reacted with avidin DH and biotinylated horseradish peroxidase H reagent [avidin-biotin complex (ABC), Vector Laboratories, Burlgame, CA, USA] for 45 minutes at room temperature. Sections were washed three times (five minutes each) in PBS. They were then incubated with 0.05% diaminobenzidine tetrahydrochloride for eight to ten minutes. Slides were washed in tap water for five minutes, counterstained with haematoxylin and mounted with a synthetic medium. Suitable positive controls for each lectin, either intrinsic or extrinsic (i.e. specific parts of the nephron and, generally, of the normal kidney), were stained in each experiment. Control procedures for lectin binding specificity consisted in incubation of the sections in labelled lectins at concentrations indicated in specific for each lectin sugar-containing solutions (in PBS). „Negative“ controls were also available by substituting PBS for each lectin in the first staining step. Slides were coded before examination so that the observers were unaware of which lectin had been used and whether pretreatment with neuraminidase had been applied. For normal mucosa and adenoma tissues, the percentage of positive crypts was determined. A section was judged to be positive when more than 5% of the crypt area showed a positive reaction.

The growth fraction was defined by three-step immunostaining for Proliferating Cell Nuclear Antigen (PCNA, Monoclonal Mouse Anti-Antibody, clone PC10, DAKO, Glostrup, Denmark). Most PCNA data were available from a previous study.[12] The percentage of PCNA positive cells was determined by counting 300 cells of each adenoma case. Counts were performed in random high power fields.

Statistical analysis

After the examination of the descriptive data, the main analysis was performed by the multiple logistic regression method. The latter is used when the dependent and independent variables are qualitative. It allows the estimation of the frequency of the dependent variable in two groups of each independent variable as well as the assessment of the predictive value of each of the independent variables. In the present study the dependent variable was the degree of dysplasia. As previously stated, the latter was categorized in two groups [low grade and high grade (precancerous): GI and GII respectively]. The independent variables examined were the following: 1. each lectin’s expression with and without neuraminidase treatment (absence of

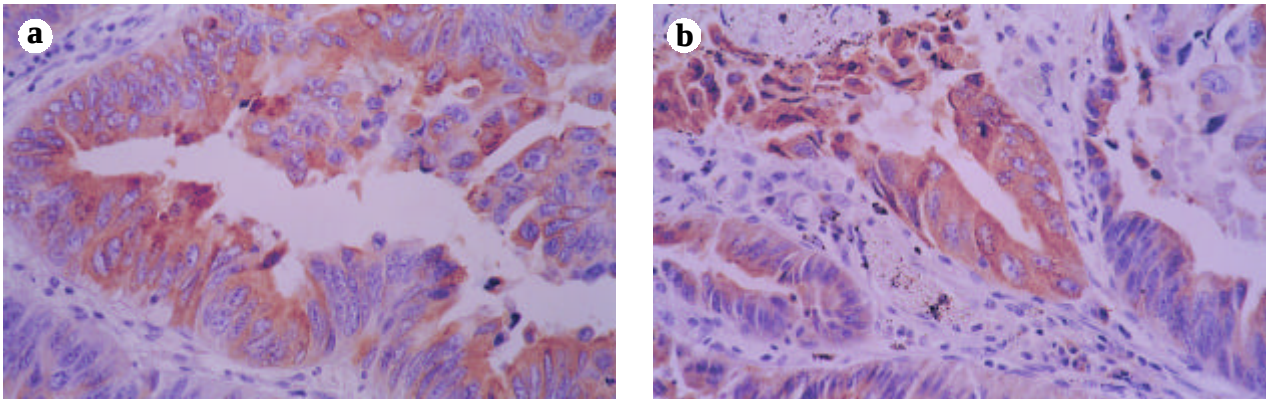

Figure 2a,b. PNA cytoplasmic positivity in a considerable number of highly dysplastic adenomatous cells (PNA histochemistry, original magnification x 400).

Table 2. **Percentages of lectin positive adenomas**

Lectin type	*N (total=52)*	*%*
PHA (+N)	34/52	65.4%
PHA (-N)	34/52	65.4%
UEA-I (+N)	18/52	34.6%
UEA-I (-N)	16/52	30.8%
WGA (+N)	18/52	34.5%
WGA (-N)	7/52	13.5%
PNA (+N)	21/52	40.4%
PNA (-N)	7/52	13.5%

lectin = 0, presence of lectin = 1), 2. gender (male = 0, female = 1), 3. patients' age. Tumor size and histologic type were excluded from the statistical model as they are well established independent predictors of the likelihood of malignancy. A p value less than 0.05 was considered statistically significant.

Results

Lectin staining

PHA and WGA showed some binding in colonic cells of the normal mucosa whereas UEA-I and PNA were practically negative. After neuraminidase treatment, however, PNA binding was seen in at least some goblets in all specimens, while UEA-I was found to be either luminal or luminal diffusely precipitated in columnar cells (and very rarely in goblet cells particularly from specimens of the right hemicolon).

In adenomas, UEA-I binding was almost exclusively directed to the luminal surface (*Figure 1*) and must thus reflect changes in structural membranic glycoproteins; it also involved intraluminal secretions. On the other hand, PNA binding was predominantly intracellular and therefore probably reflects a change in mucin synthesis within the Golgi apparatus. In detail, PNA was most commonly located in the supranuclear regions of goblet and non goblet (columnar absorptive) cells and occasionally within the mucin goblet itself but not within the apical cytoplasm of columnar absorptive cells. Regardless of the kind of lectin, positivity in adenomatous crypts with high grade of dysplasia was always of the diffuse pattern with lectins reacting strongly with cytoplasmic constituents (*Figure 2a,b*).

Statistical correlations

Table 2 summarizes the proportion of adenomas which were found positive for each of the examined lectins with or without neuraminidase treatment. PHA was expressed in the greatest proportion of specimens (65.4%), irrespective of prior neuraminidase treatment of the sections. None of the examined lectins was influenced by the adenoma' s size. We found no correlation between location of the lesion and probability of detection by any lectin.

Table 3 demonstrates the values of regression coefficients (β) and their confidence intervals, examining whether the dysplasia grade is dependent on the expression of each lectin, as well as on patients' gender and age.

From the model containing PHA as an independent variable, we can see that the frequency of high grade dys-

Table 3. **Regression coefficient β (95% CI) of degree of dysplasia with regard to the four examined lectins expression with and without neuraminidase pretreatment, patients' gender and age.**

a.	β *(95%CI)*	*p-value*	β *(95%CI)*		*p-value*
a. Lectin PHA					
age	1.13(1.05-1.22)	**0.001**	age	1.09(1.02-1.17)	**0.01**
gender	3.75(0.88-15.95)	0.074	gender	3.24(0.75-13.88)	0.11
PHA(+N)	4.19(0.93-18.91)	**0.062**	PHV(-N)	0.31(0.07-1.40)	0.12
b. Lectin UEA-I					
age	1.13(1.03-1.23)	**0.07**	age	1.16(1.05-1.27)	**0.002**
gender	2.39(0.62-9.27)	0.20	gender	3.24(0.73-14.36	0.12
UEA-I(+N)	1.76(0.29-10.48)	0.53	UEA-I(-N)	12.71(1.96-82.25)	**0.007**
c. Lectin WGA					
age	1.12(1.02-1.24)	**0.02**	age	1.11(0.96-1.19)	**0.004**
gender	2.52(0.65-9.74)	0.18	gender	2.47(0.64-9.51)	0.18
WGA(+N)	1.36(0.20-9.73)	0.75	WGA(-N)	1.26(0.12-13.26)	0.85
d. Lectin PNA					
age	1.09(1.01-1.17)	**0.01**	age	1.13(1.05-1.22)	**0.001**
gender	2.84(0.72-11.1)	0.13	gender	1.78(0.42-7.54)	0.42
PNA(+N)	2.95(0.72-12.02)	0.13	PNA(-N)	0.21(0.03-1.48)	0.11

Table 4. **Observed and expected cases with dysplasia of low and high grade with regard to the four examined lectins expression with and without neuraminidase pretreatment, as well as to patients' gender and age.**

a. Lectin	*PHA(+N)*		*PHA(-N)*		
			Expected cases		
	GI	GII	GI	GII	
Observed cases					
GI	15	8	23 (65.2%)	14	9	23 (60.87%)
GII	6	23	29 (79.3%)	6	23	9 (79.31%)
	21	21	52	20	32	52
Total predictive value: 73.08%			Total predictive value: 71.15%			

b. Lectin	*UEA-I(+N)*		*UEA-I(-N)*		
			Expected cases		
	G	GII	GI	GII	
Observed cases					
GI	13	10	23 (56%)	16	7	23 (69%)
GII	6	23	29 (79%)	2	27	29 (93%)
	19	33	52	18	34	52
Total predictive value: 69%			Total predictive value: **83%**			

c. Lectin	*WGA(+N)*		*WGA(-N)*		
			Expected cases		
	GI	GII	GI	GII	
Observed cases					
GI	13	10	23 (56%)	13	10	23 (56%)
GII	6	23	29 (79%)	5	24	29 (83%)
	19	33	52	18	34	52
Total predictive value: 69%			Total predictive value: 71%			

d. Lectin	*PNA(+N)*		*PNA(-N)*		
			Expected cases		
	GI	GII	GI	GII	
Observed cases					
GI	14	9	23 (61%)	15	8	23 (65%)
GII	6	23	29 (79%)	7	22	29 (79%)
	20	32	52	22	30	52
Total predictive value: 71%			Total predictive value: 71%			

plasia (GII) in colorectal adenomas is 30% greater than that of low grade dysplasia (GI) for each year of age, 3.75 times more frequent in women compared to men (though at statistically just suggestive level of significance $p = 0.074$) and 4 times more frequent in PHA(+N) – expressing adenomas. Interestingly, of all examined lectins with neuraminidase pretreatment, only PHA showed a tendency for association with grade of dysplasia; nevertheless, this association could not reach statistical significance ($p = 0.062$, *Table 3a*). As concerns PHA(-N), the frequency of high grade dysplasia in PHA(-N) expressing adenomas is three times less than that of the rest . For this specific lectin, the correct prediction percentages of degree of dysplasia based on patients' gender, age as well as PHA(+N) and (-N) expression were 73% and 71% respectively (*Table 4a*). The correct prediction of high grade dysplasia is the same in both models (79.3%) irrespective of neuraminidase treatment. On the contrary, the prediction of low grade dysplasia is better when neuraminidase treatment is performed (65.2%, by contrast with 60.9%, *Table 4a*).

In the model containing UEA-I as an independent variable, patients' age is again positively linked to dysplasia grade at a statistically significant level and the same counts for UEA-I(-N) expression (*Table 3b*). It is worth noting than when UEA-I(-N) expression is assessed as an independent variable, the correct total prediction of dysplasia rises to a percentage of 83% (*Table 4b*).

Table 5. **Correct prediction of adenomas' grade of dysplasia based on the assessed models.**

	Correct prediction of dysplasia		
Model for lectin	*GI*	*GII*	*In total*
PHA(+N)	65%	79%	73%
PHA(-N)	61%	79%	71%
UEA-I(+N)	56%	79%	69%
UEA-I(-N)	69%	**93%**	**83%**
WGA(+N)	56%	79%	69%
WGA(-N)	56%	83%	71%
PNA(+N)	61%	79%	71%
PNA(-N)	65%	79%	71%

As concerns the models containing WGA and PNA, age is the only factor found to be positively associated with the grade of dysplasia (*Table 3c, 3d*). WGA staining is useful in predicting high grade dysplasia in quite a lot of adenomas but, again, quite a lot of low grade dysplastic specimens are missed (*Table 4*).

In summary, in all models examined, age was always positively correlated to degree of dysplasia at a statistically significant level; in other words, as age increases the possibility of high grade dysplasia also increases. Only UEA-I positivity without neuraminidase treatment [UEA-I (-N)] also managed to achieve a significant association with grade of dysplasia ($p = 0.007$, *Table 3*). According to table V, the model which better predicts grade of dysplasia is the one which, in addition to patients' age and gender, takes UEA-I(-N) expression into account. However, the latter model is obviously more helpful in predicting high grade dysplasia (93%) than low grade dysplasia (69%). In general, low grade dysplasia is not so well predicted by lectin staining as high grade dysplasia.

Cell kinetics

The PCNA score for adenomas of this study ($n = 52$) ranged from 8% to 80% (mean ± SEM: 36.1%±3.2%, 95% CI: 29.65–42.54). In particular, adenomas with UEA-I positive labelling were characterized by higher proliferation capacity of the neoplastic cells (PCNA scores >40%) compared to the rest, at a statistically significant level ($p<0.05$).

Discussion

It is accepted that the process of colon tumorigenesis can affect the binding of neoglucoproteins;[13] in general, glycoconjugate modifications are early events in the evolution of the neoplastic phenotype.[14] Moreover, given that lectins bind to mucins, and that changes in mucin production will depend on the specific mutations that spawn each polyp, it stands to reason that not all lesions will produce the same abnormal mucin. Therefore, not all neoplasms would necessarily be expected to bind a given lectin[15]. Areas of high grade dysplasia consist mainly of non mucin columnar cells whereas the background adenoma tends to be composed mainly of goblet cells. Most colorectal cancers do not produce mucin goblets either, hence, recapitulating undifferentiated features; let us point out that the ability of colonic epithelial cells to produce mucin is indicative that these cells are differentiated cells. This information on altered glycosylation and mucin production detected by lectin binding, prompted us to examine the staining patterns of four well known lectins in a series of adenomatous polyps. In particular, PNA, UEA-I and WGA were chosen based on literature showing specificity of the above lectins for dysplastic mucosa.[13,15-17] The study was safely performed on archival material as previous studies have demonstrated that lectin binding characteristics are stable in colonic tissues stored in formalin for up to 2 months before paraffin embedding, and there are no changes in tissue glycoconjugates stored for years after embedding in paraffin.[18]

It has been shown that PNA binds more avidly to mucins from colon cancer than to those from normal colon.[19]In our study, both UEA-I and PNA showed very little binding to normal colonic cells and only when some of their glycoconjugate sequences were unmasked by neuraminidase. So, these two lectins without neuraminidase treatment can be considered as markers of the adenomatous epithelium. Furthermore, each one of them appears to possess a distinct binding pattern related to separate biologic functions in differentiated goblet or columnar cells. In highly dysplastic cells, however, both lectins demonstrate diffuse binding pattern throughout the cytoplasm (*Figure 2*). These different staining patterns are probably related to the degree of cellular differentiation since in the process of malignant transformation the carbohydrate distribution undergoes progressive changes through the adenoma-carcinoma sequence. The latter are related to the degree of dysplasia in adenomas. Low grade dysplasia could not be safely predicted by any of the examined lectins in the examined samples. We must also take into account that there is a shortcoming when evaluating lectin labeling. Results may lack absolute precision since most of the lectins used are not monospecific but have an affinity for more than one mono- di- or tri- saccharides; their binding to a particular sugar does not simply depend upon its presence but also on the position of outer sugars in relation to it in the glycan chain.[11] So, although speculations can be made about the correlation between composition of glycoproteins expressed in the adenomatous cells and lectin binding, no specific conclusion can be inferred without additional, biochemical studies.

It is of interest that among all lectins evaluated, UEA-I was more clearly associated with high grade dysplasia and

this may be related to the same lectin' s significant association with high proliferation rate of neoplastic cells. Lectin-binding changes such as the UEA-I binding in adenomatous epithelial cells may actually precede the increased cell proliferation (as evidenced in the present study by high PCNA scores), thus probably corresponding to an earlier event in the carcinogenesis chain.[20] Many lectins are potent exogenous growth signallers, mimicking the action of metabolic hormones. This biological activity is a function of lectin structure through recognition and binding to specific carbohydrate receptors expressed on cellular membranes. In some instances, internalization of the lectin may be required[7]. Lectin receptor expression, as evidenced indirectly by lectin histochemical binding, also appears to be increased in neoplastic colonic mucosa allowing interaction with certain dietary lectins which are resistant to digestion and which would otherwise pass through the normal colon without binding. Assuming that some of these lectins possess a mitogenic effect, changes in lectin receptor expression and subsequent lectin binding could effect regulation of epithelial growth and subsequent malignancy. Although increased growth effects have so far been attributed to the mitogenic effect of PNA,[7] the present study implies a similar role for UEA-I in the proliferative response of colonic cells during tumorigenesis.

In conclusion, colorectal adenomas and especially the highly dysplastic ones show changes in lectin-binding patterns in histological specimens. UEA-I binding without neuraminidase treatment is closely associated with high grade of dysplasia and high proliferation rate of the neoplastic cells. This interesting observation, however, requires verification on a larger scale before acquiring some clinical utility; for instance, in the study of exfoliative cells from bowel mucosa. On the other hand, let us point out that age was the only factor which showed a constant positive association with high grade of dysplasia.

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