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An Immunohistochemical Study of PNA (peaunt agglutinin) Binding in Transitional Cell Carcinomas of the Urinary Bladder

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INTRODUCTION

Lectins are divalent or multivalent or multivalent carbohydrate-binding proteins of defined specificity and have been extensively used in the study of cell surface phenomenon[1-3].

Fluorescein (−), peroxidase (−), or avidin-biotin complex-labelled lectins applied as histochemical reagents have provided valuable data on both normal and pathologically altered tissues[4-7], however, the data gathered so far remain controversial and it is still not clear whether they could be used in histopathology, either to increase diagnostic accuracy or to understand the histogenesis of tumors.

Investigations of epithelial cell surfaces of urinary bladder neoplasia by lectins, particularly transitional cell carcinomas, have been few[8] but there are some evidences of strong binding of the peanut agglutinin (PNA) in transitional cell carcinomas of the urinary bladder[8,9].

The authors attempted the PAN binding study of normal mucosa, von Brunn's ness, cystitis cystica, and transitional cell carcinomas of the urinary bladder by avidin-biotin complex method to know whether PNA immunohistochemistry could facilitate the accurate diagnosis and/or grading of anaplasia of transitional cell carcinomas of the urinary bladder.

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MATERIALS AND METHODS

1. Materials (Table 1)

Buffered formalin-fixed, paraffin-embedded tissues of ten normal mucosa of the urinary bladder, four von Brunn's nests and cystitis cystica, and sixty-one transitional cell carcinomas of the urinary bladder were examined. The transitional cell carcinomas were divided into grade I to IV according to Ash's grading system[10]. Among sixty-one transitional cell carcinomas in this study, Ash grade I, II, III and IV were ten, twenty-two, twenty, and nine cases, respectively.

2. Staining procedures

The staining procedures were as follows:

1) Sections were cut 4 um in thickness and incubated in 56℃ for 30 minutes.

2) Slides were deparaffinized with xylene and rehydrated with graded ethanols.

Table 1. Materials

Diagnosis	No. examined
Normal mucosa	10
Von Brunn's nest & cystitis cystica	4
TCC*, grade I	10
TCC, grade II	22
TCC, grade III	20
TCC, grade IV	9

* TCC : Transitional Cell Carcinoma

3) Following rehydration, the sections were incubated with 4% hydrogen peroxide in methanol for 30 minutes.

4) After washing (3 times 3 minutes) in Tris-buffered saline, pH 7.4, the sections were incubated with 1:20 diluted normal goat serum (Vectastain, PK3001) for 30 minutes.

5) The sections were then incubated with 1:200 dilution of biotinylated PNA (Vector Lab. Inc) for one hour.

6) Following incubation, the slides were washed 3 times 5 minutes in Tris-buffered saline, pH 7.4.

7) And avidin-biotin complex (Vectastain, PK4001) was added to the sections for 30 minutes. The used ABC was prepared by avidin solution 1 drop and biotinylated peroxidase solution 1 drop in addition to 5 ml Tris-buffered saline.

8) After washing with Tris-buffered saline, the color was developed with 3,3'-diamino-benzidine: hydrogen peroxide (Sigma Chemical Company) for 5 to 10 minutes.

9) After washing with tap water, the slides were counterstained with Mayer's hematoxylin for 2 minutes, dehydrated in a series of graded ethanols, cleared in xylene, and coverslipped with balsam mounting.

3. Interpretation

The positive areas were stained brown to dark brown in color. And the degrees of PNA binding in positive cases were classified as:

1) Focal (10% or less of surface areas expressing PNA binding),

2) Moderate (11%～49% of surface areas expressing PNA binding),

3) Diffuse (50% or more of surface areas expressing PNA binding).

These estimations and determinations were done by two pathologists as unknowns to heighten the objectivity.

RESULTS

The patterns of PNA staining in ten specimens from the normal urinary bladder mucosa, four specimens of von Brunn's nests and cystitis cystica, and sixty one specimens from transitional cell carcinomas of the urinary bladder are summerized in Table 2.

The epithelium of normal mucosa showed negative staining for PNA (Fig. 1) in all but two specimens where focal staining of glycocalyx of umbrellar cells (Fig. 2) are noted. PNA did not stain blood vessels and connective tissues in the stroma except red blood cells. Four cases of von Brunn's nests and cystitis cystica were negative for PNA staining. In transitional cell carcinomas, the numbers of positive cases of PNA binding are increased with Ash grade I to IV, 20% (2/10 cases), 41% (9/22), 55% (11/20) and 88% (8/9), respectively. The positive reaction patterns are observed along the cell membrane and in the cytoplasm (Fig. 3).

Table 2. PNA staining patterns of the normal mucosa, von Brunn's nest, cystitis cystica, and transitional cell carcinoma of the urinary bladder

Diagnosis	No. of specimen	No. of positive staining	Patterns of staining
Normal mucosa	10	0 (0%)	the uppermost cell border (glycocalyx)
von Brunn's nest and cystitis cystica	4	0 (0%)	
TCC* (I)**	10	2 (20%)	intracytoplasm & cell membrane
TCC (II)	22	9 (41%)	"
TCC (III)	20	11 (55%)	"
TCC (IV)	9	8 (88%)	"

* TCC : Transistional Cell Carcinoma, ** ( ) : Ash grade I to IV.

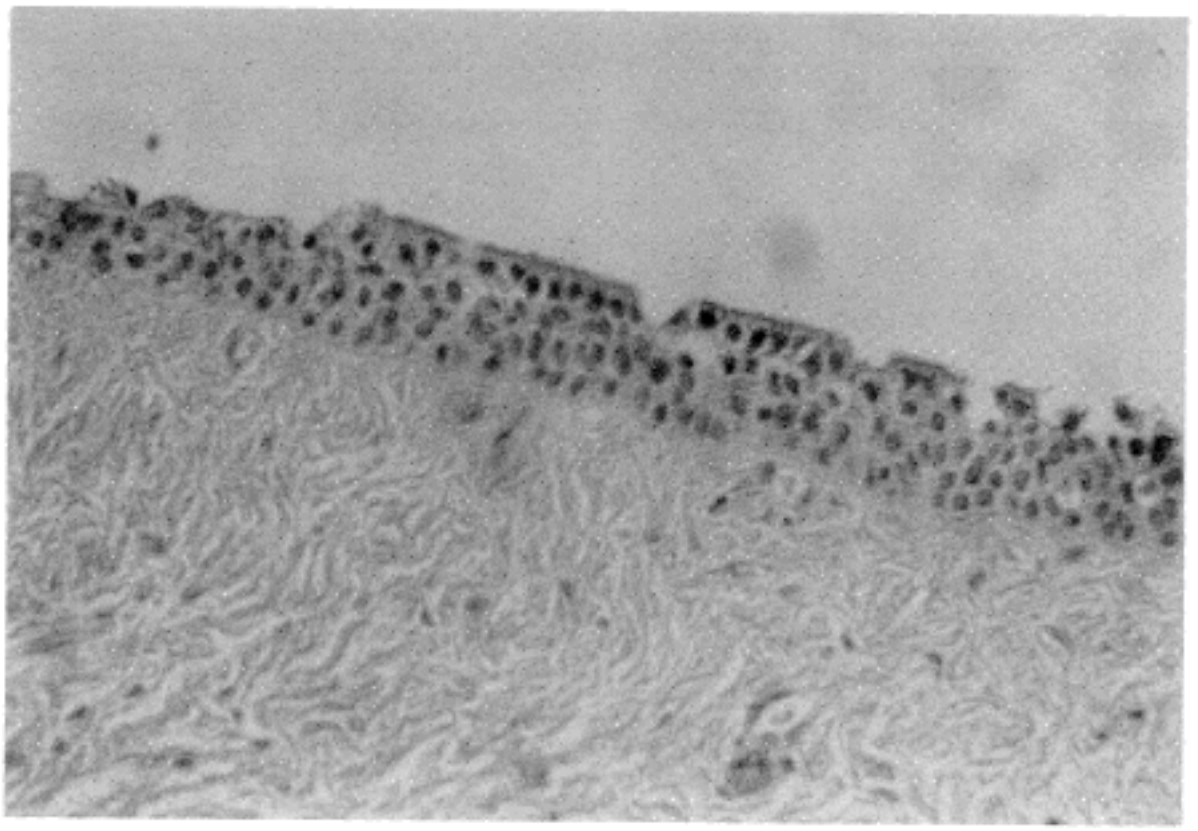

Fig. 1. Negative PNA staining of normal mucosa of the urinary bladder (PNA ABC, ×250).

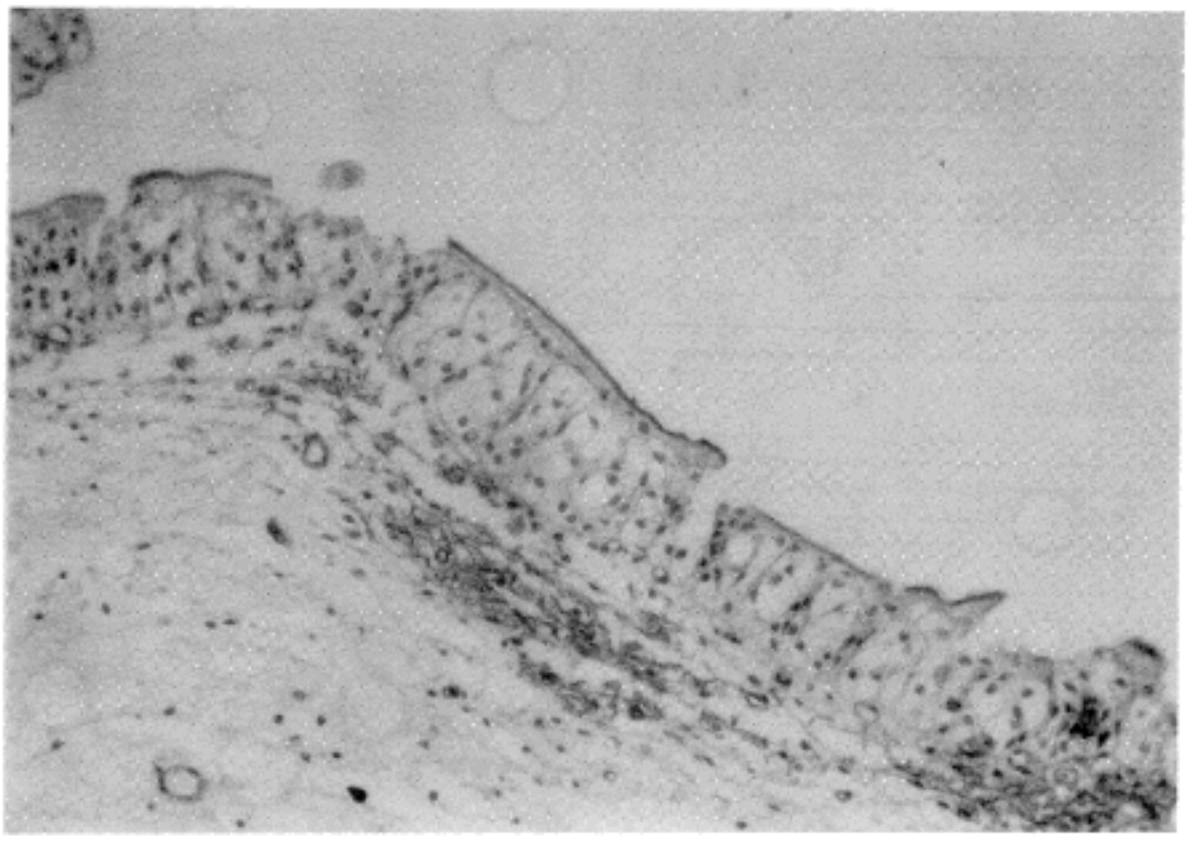

Fig. 2. Positive PNA staining on the luminal border of umbrellar cells (PNA ABC, ×250).

Chi-square test revealed statistically significant difference of the positivity of PNA binding between the normal mucosa and transitional cell carcinomas of Ash grade I to IV ($p<0.05$).

Degree of PNA bindings in transitional cell carcinomas were classifed as focal (Fig. 4), moderate (Fig. 5) and diffuse (Fig. 6) by light microscopic examination(Table 3). Although Table 3 showed that the degree of PNA binding appears to be increased with Ash grade I to IV in transitional cell carcinomas, however, it did not show statistically significant difference ($p>0.05$).

DISCUSSION

Lectins are carbohydrate-binding proteins[2,4],

Fig. 3. Diffuse PNA staining of transitional cell carcinoma, grade IV. Note the membranous and cytoplasmic staining patterns (PNA ABC, ×100).

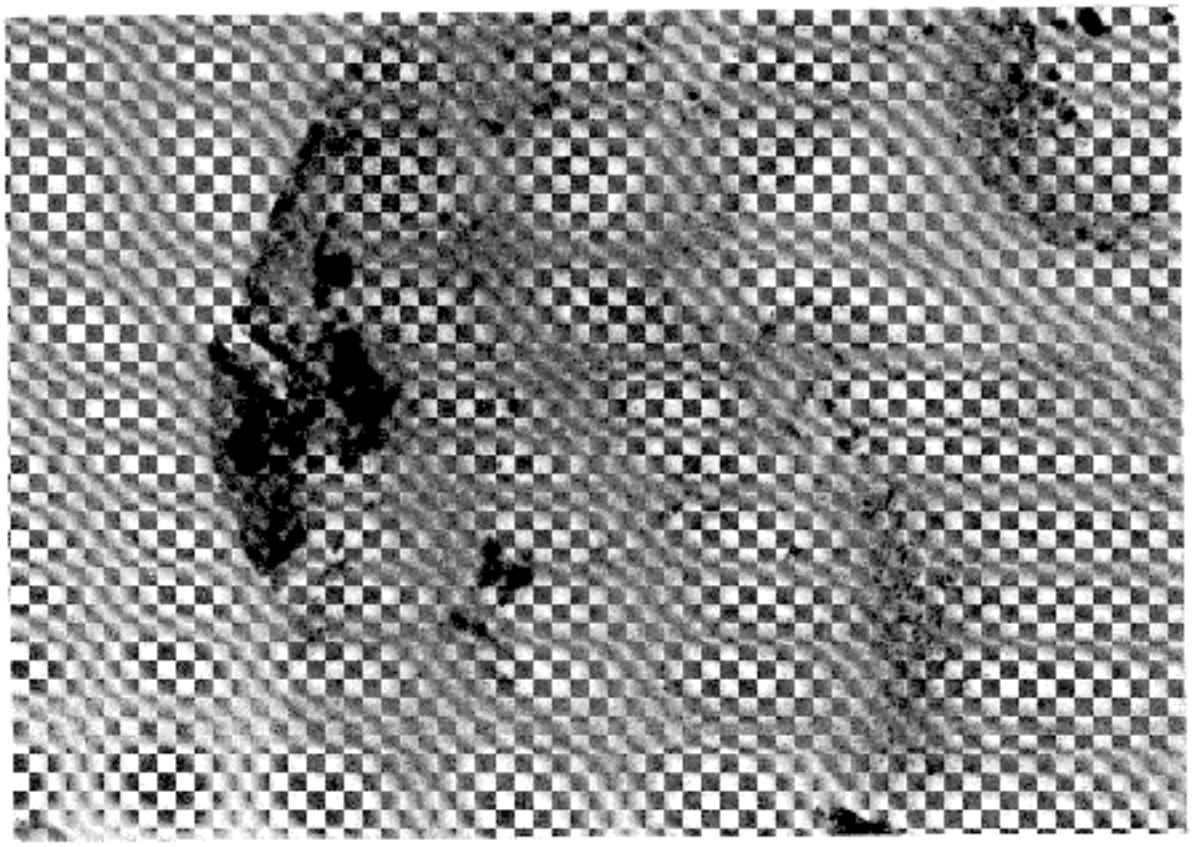

Fig. 4. Focal PNA staining of transitional cell carcinoma, grade I (PNA ABC, ×100).

which have an affinity for certain specific sugars and recently they have been used to probe the structural features of cell surface glycoproteins[1-3] and to investigate the role of the cell surface changes in cellular functions such as growth, adhesion, antigenicity, pinocytosis and differentiation[4-6].

It seemed possible that specific lectins could be found that would associated with the cell surface of malignant tumors if changes in surface carbohydrates occured. Extensive use has been made of lectins as cytochemical markers through horseradish peroxidase conjugates, fluorscein isothiocyanate conjugates, or avidin-biotin complexes[4,5,7].

Since the cell surface changes appear to be important in the development of malignant neoplasia, the

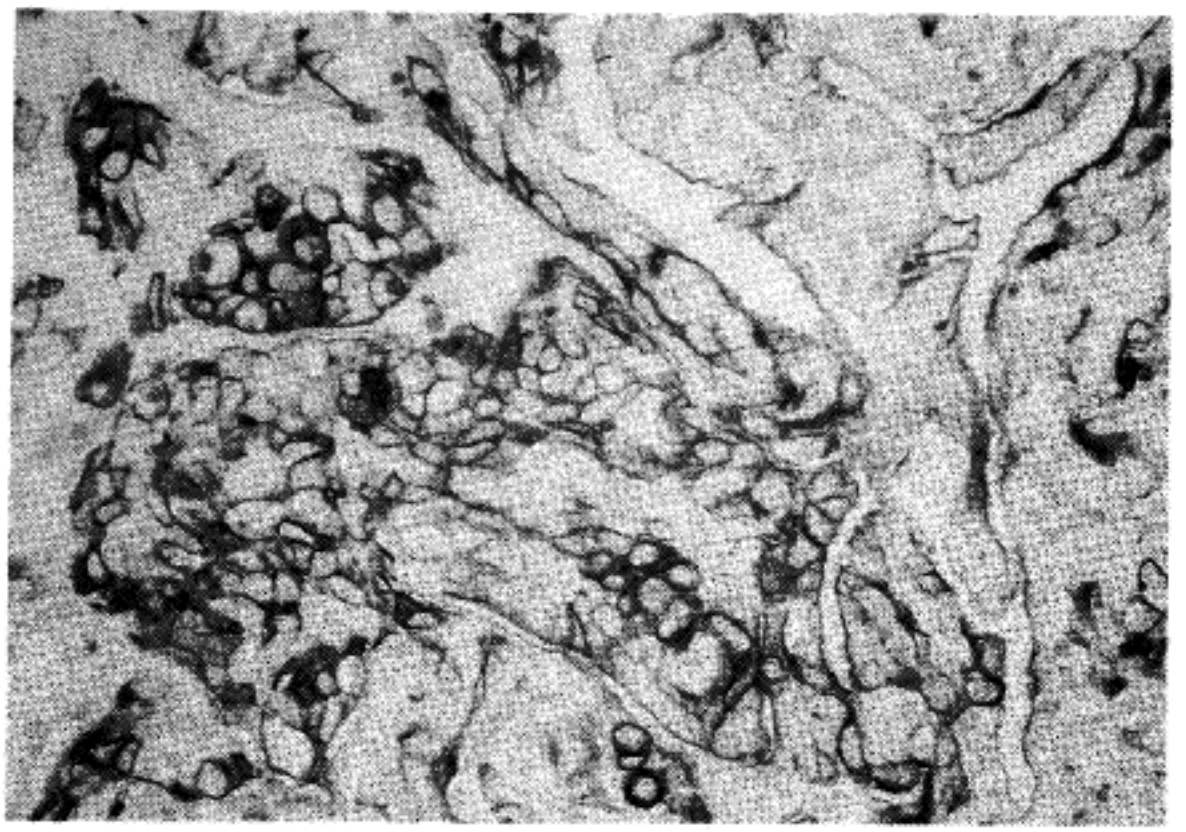

Fig. 5. Moderate PNA staining of transitional cell carcinoma, grade II (PNA ABC, ×100).

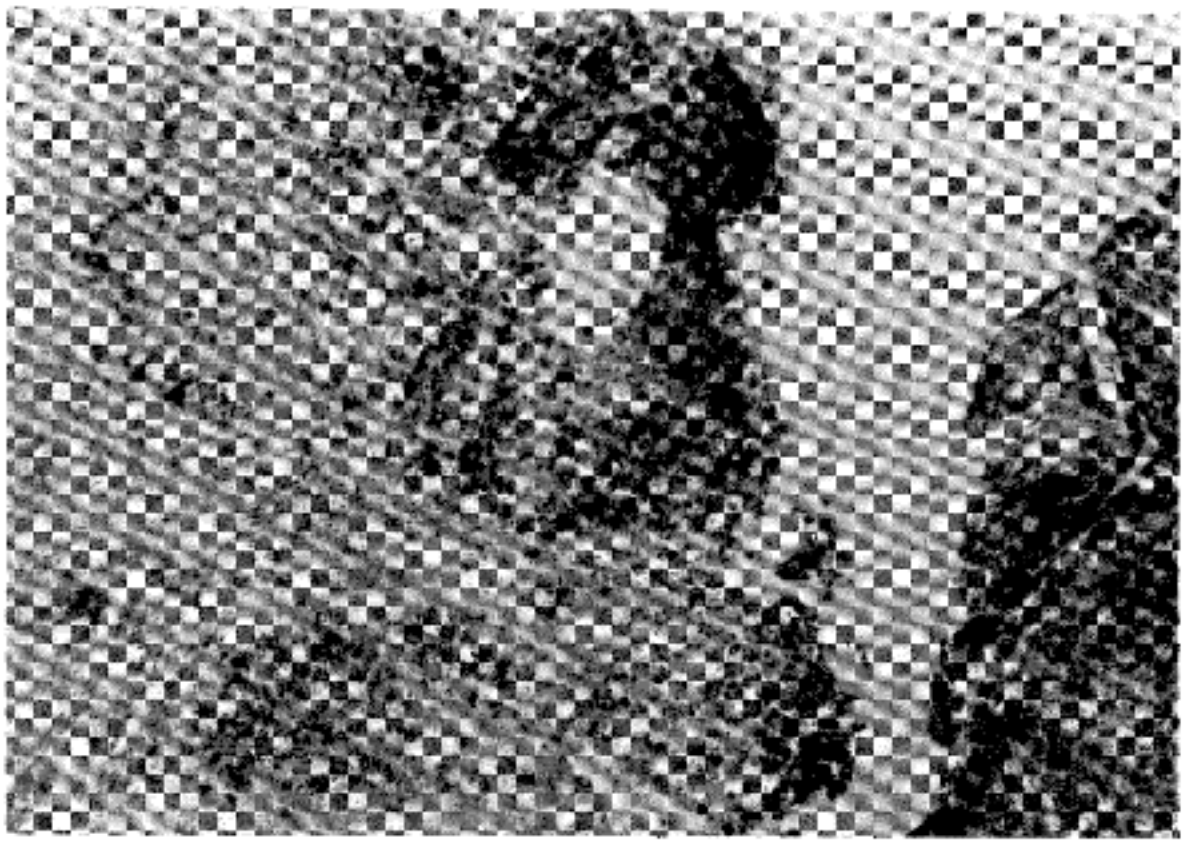

Fig. 6. Diffuse PNA staining of transitional cell carcinoma, grade III (PNA ABC, ×100).

elucidation of cell surface properties may well allow a more sensitive and reliable assessment for diagnosis and as a predictor of tumor behavior such as recurrence and metastasis. Thus, informations about the cell surface and its glycoprotins and glycolipids can readily be obtained using lectins.

This PNA (peanut agglutinin) histochemical study had two principal aims: (1) to define the changes in the cell surface glycoconjugates that could be helpful in more accurate and early histologic diagnosis of transitional cell carcinomas of the urinary bladder and (2) to determine whether PNA histochemistry could be used in histological grading of differentiation.

To provide a baseline and reference point for the study of tumors, I have first studied the PNA binding

Table 3. Degree of PNA bindings of the normal mucosa, von Brunn's nest, cystitis cystic-, and transitional cell carcinomas of the urinary bladder

Degree of binding	Normal mucosa	von Brunn's nest & cyct-itis cystica	TCC (I)	TCC (II)	TCC (III)	TCC (IV)
Focal	0	0	1	3	4	2
moderate	0	0	1	4	5	2
diffuse	0	0	0	2	2	4
positive cases	0	0	2	9	11	8
Total Cases	10	4	10	22	20	9

in normal urinary bladder mucosa. In normal mucosa, PNA (specific for D-galactose residues) stained partly luminal surfaces of umbrellar cells in two specimens and all ten specimens were negative for PNA staining.

Ten grade I transitional cell carcinomas showed focal to moderate degree of PNA binding in two cases (20%) and that positive patterns were intracytoplasmic and/or membranous.

Grade II transitional cell carcinomas showed the similar staining patterns to grade I except relatively increased percentage of positive PNA binding cases, which was 41% (9/22 cases). Among nine positive cases, the degree of PNA binding revealed focal in three, moderate in four and diffuse in two cases.

In cases of grade III and IV transitional cell carcinomas, the staining patterns were similar to grade I or II but the degree of binding was more increased than that of grade I or II cases, especially in poorly differentiated tumor cells. Percentages of positive cases of PNA binding of grade III and IV transitional cell carcinomas were 55% (11/20 cases) and 88% (8/9 cases), respectively.

The plasma membrane of the transitional epithelium of the urinary bladder exhibits ultrastructurally unique membrane plaques[9,11~13]. With extensive studies[14~18], lipid and protein compositions of the membrane structure were elucidated, but its carbohydrate moieties have not completely defined yet.

This study has used PNA, which has an affinity for the disaccharide D-galactose beta[1~3] D-N-acetyl-galactosamine, which is an antigenic determinant for the Thomson-Friedenreich antigen (TAg)[19,20]. Numerous studies have been reported the prognostic significance of the ABH blood group antigens in carcinomas of the urinary bladder[21~26]. This is accompanied by the appearance of precursor or cryptic antigens concomitant with disappearance of normal antigens. One of the defined precursor determinants is the T antigen, which is expressed in many carcinomas[20], but not in a number of normal epithelia such as transitional epithelium[27]. This TAg is usually covered by a terminal sialic acid. Some investigators have claimed that this TAg is tumor specific, whereas others have described a cytostructural relocalization of this antigen.

Regarding urothelial neoplasm, Limas and Lange[22] noted that four of six cases of invasive transitional cell carcinomas expressed TAg while all controls failed to express TAg. Coon and associates[27] noted that grade III, grade I and II transitional cell carcinomas expressed 67% and 21% positivity for TAg (PNA binding). They also noted that grade I and II transitional cell carcinomas were cryptic-TAg-positive. Lehman and associates[8] noted that only 47% of grade II transitional cell carcinomas expressed TAg. More recently, Alroy and associates[30] have also shown that non-neoplastic transitional epithelium failed to express the TAg without prior neuraminidase treatment. However, in our study, two specimens of normal urinary bladder mucosa expressed PNA-binding at the cell surface coat without prior treatment of neuraminidase. This result may be considered that formailn-fixed paraffin-embedded tissues and tissue processing may have affected carbohydrate preservation. But, none of ten normal mucosa showed PNA binding in the cytoplasm and/or the cell membrane. Four cases of von Brunn's nests and cystitis cystica are negative for PNA staining, which may seemed to be the similar nature to normal mucosa in these mataplastic transitional epithelium.

The results obtained from transitional cell carcinomas in this study suggest that simultaneous membranous and intracytoplasmic bindings of PNA in tumor cells represent rather constant features of

these cells. In cases of focal or patch PNA staining in transitional cell carcinomas, it may be due to heterogeneity within individual tumors. Coon et al[27] and Lehman et al[8] found focal staining of transitional cell carcinomas and Thompson et al[31,32] using monoclonal antibodies in immunohistologic techniques have found that stainings within individual tumors often were focal.

As noted by Springer and associates[28], the TAg is considered to be a precursor of the MN blood group system. The synthesis of the MN blood group glycoprotein follows a progression in which the protein backbone is synthesized in the rough endoplasmic reticulum, and the sugar moieties are added sequentially by membrane bound transferase in the Golgi apparatus, which would be responsible for cytoplasmic staining. During malignant transformation, urothelial barrier is compromised and concomitantly cell surface changes occur and it may also be expected the changes or simplification of cell surface carbohydrates.

Thus, the expression of the TAg in transitional cell carcinomas most likely represents an incomplete synthesis of the MN blood group glycoproteins, with absence of the terminal sialic acid.

In Chi-square test, statistical significance is noted between percentage of PNA positive cases of normal bladder mucosa and transitional cell carcinomas and also between grades of transitional cell carcinomas ($p<0.05$).

Degree of PNA binding (or PNA receptor) of transitional cell carcinomas to Ash grades is slightly increased, but is not significant statistically ($p>0.05$).

Based on this study, transitional cell carcinomas of urinary bladder more easily morphologically identified by PNA-binding assay. Therefore, I recommend this assay as a simple and quick tool for routine diagnostic evaluation of suspected transitional cell carcinoma of the urinary bladder, especially in controversial cases. However, the degree of differentiation or anaplasia of transitional cell carcinomas have not been strictly applied by PNA binding assay. This study did not reveal the relationship between PNA bindings and invasiveness of tumor cells and other biological behaviors. Therefore, further studies for these aspects and also chemical analysis of structures of PNA binding sites (i.e. PNA receptors) are necessary.

CONCLUSIONS

Recently, extensive uses of lectins as cytochemical markers have made of studies for various epithelial and nonepithelial neoplasia, however, investigations of epithelial cell surface of transitional cell carcinomas of the urinary bladder have been few. Thus, the authors performed a study of PNA binding in transitional cell carcinomas with comparision with that in normal mucosa of the urinary bladder to allow more accurate diagnosis and histological grade or degree of differentiation.

The results of this study are as follows:

1) PNA shows negative reactions on all ten normal mucosae of the urinary bladder but positive staining at the glycocalyx of umbrellar cells in two cases.

2) PNA shows negative reactions on all four cases of von Brun'n nests and cystitis cystica.

3) PNA shows positive reactions on thirty (50%) of total sixty-one cases of transitional cell carcinomas and reveals two (20%), nine (41%), eleven (55%) and eight (88%) cases in grade I, II, III and IV, respectively.

4) PNA shows positive reactions on the intracytoplasm and/or degree of PNA binding activity in grade I to IV transitional cell carcinomas is not statistically significantly different ($p>0.05$).

In summary, PNA did not react with normal nucosa and metaplastic lesions such as von Brunn's nests and cystitis cystica, however, it reacted with 50% (30/61 cases) of transitional cell carcinomas and its positivity is significantly increased with gradings of transitional cell carcinomas ($p<0.05$).

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═국문초록═

방광의 이행상피암종에 대한 PNA (peanut agglutinin) 결합에 관한 면역조직화학적 연구

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김철환 · 원남희 · 이갑노 · 백승룡

최근에 식물응집소들이 2가 또는 다가 다당류의 탄수화물기에 특이하게 결합하는 성질을 갖는 당단백질이라는 것을 이용하여 정상 및 암조직 세포에 대한 결합력의 존재 여부 및 그 변화에 관한 연구들이 행해지고 있다. 그러나 아직까지 이러한 연구결과들 사이에는 논쟁의 여지가 있어서, 실제로 병리의사가 이들 식물응집소들을 병리학적 조직의 진단 등에 응용하기에는 어려운 점이 많다. 따라서 좀더 폭넓게 이들 식물응집소들을 이용하기 위해서는 이에 대한 연구가 더 이루어져야 한다. 저자는 방광암에 대한 식물 응집소 결합에 관한 연구가 거의 없고, 특히 방광암에서는 혈액군 동종항원의 변화가 그 종양의 생물학적 특성, 즉 재발 및 전이와 유의한 관계가 있기 때문에 식물응집소의 결합력의 변화와도 밀접한 관계가 있을 것이라는 가설을 뒷받침하기 위해 다음과 같은 실험을 하였다.

실험재료로서 정상 방광 점막, 화생성 병변인 Brunn씨 세포소 및 낭포성 방광염, 그리고 방광 이행상피암을 대상으로 하여 이에 대한 PNA(Peanut agglutinin)의 결합 형태와 결합 정도를 조사하고 이를 통계학적으로 비교 분석하여 PNA에 의한 이행상피암종의 진단이나 분화도에 대한 조직병리학적 응용 가능성을 알아보았다.

사용한 조직은 파라핀 포매된 포르말린 고정의 10예의 정상 방광 점막, 4예의 Brunn씨 세포소 및 낭포성 방광염, 그리고 61예의 방광 이행상피암이며, 이행상피암은 Ash씨 분화도를 기준으로 하여 나누었다.

본 연구의 결과를 요약하면 다음과 같다.

첫째, PNA는 정상 방광 점막 10예에서 모두 PNA에 음성 반응을 보였으나, 2예에서는 점막 최상층의 glycocalyx에 양성반응을 나타내었다.

둘째, Brunn씨 세포소와 낭포성 방광염 4예에서 PNA는 모두 음성이었다.

셋째, 이행상피암 61예중 PNA 양성은 30예(50%)였고, 이를 분화도에 따라 Ash grade별 PNA 양성율을 보면, grade I, II, III 및 IV에서 각각 20%, 41%, 55% 및 88%였다.

넷째, 이행상피암에서 PNA 양성반응은 세포질과 세포막에 모두 나타났으며, 세포 분화도에 따른 PNA 양성 정도는 분화가 나쁠수록 보다 미만성으로 염색이 되는 경향이 있었으나 통계학적인 의미는 없었다.

이상의 결과를 종합하면, PNA는 정상 및 화생성 점막 세포에는 결합하지 않으나, 암세포에서는 약 50%에서 결합하며, 또한 암세포의 분화도가 나쁘면 나쁠수록 PNA 양성율이 높음을 알 수 있었다($p<0.05$).

Key Words: Transitional cell carcinoma, Immunohistochemistry, PNA