Pharmaceutical Research, Vol. 25, No. 2, February 2008 (© 2007)
DOI: 10.1007/s11095-007-9330-4

Research Paper

The Metastatic Stage-dependent Mucosal Expression of Sialic Acid is a Potential Marker for Targeting Colon Cancer with Cationic Polymers

Abdel Kareem Azab,[1] Jackie Kleinstern,[1] Morris Srebnik,[2,3,4] and Abraham Rubinstein[1,3,4,5]

Received February 28, 2007; accepted April 30, 2007; published online October 25, 2007

Purpose. Locoregional recurrence is the most common complication after adenocarcinoma resection in the colon, despite adjuvant chemotherapy. Therapy efficacy could be improved if designed to target malignant cells by incorporating specific recognition factors in the drugs or the drug vehicles. The aim of this study was to elucidate whether the overexpression of sialic acid (SA) on colonic malignant tissues could be utilized for drug targeting by cationic polymers.
Materials and Methods. Cell lines (IEC-6, SW-480 and SW-620) and colon polyps and normal adjacent tissues harvested from dimethylhydrazine (DMH) induced rats were used as *in vitro* and *in vivo* models of different metastatic stages of colon cancer. SA expression was identified by fluorescent wheat germ agglutinin (WGA), and verified by pretreatment with neuraminidase. The role of mucus in the mucosal binding experiments was explored by pretreatment with dithiothreitol (DTT). The binding of FITC labeled cationic polymers of various degrees of cationization to normal and malignant colonic cells and tissue was measured.
Results. SA was overexpressed on malignant colonic cells and tissues, and its expression correlated to the metastatic stage *in vitro*. The binding of the cationic copolymers to the cell lines and tissues correlated with the charge density of the polymer and with the metastatic stage of the cell line. The interaction between the malignant colonic cells and tissues with the polymers was SA dependent and increased after mucus removal.
Conclusion. Cationic polymers could be used as a targeting tool to colonic malignant epithelium, to be implemented in drug delivery and diagnosis.

KEY WORDS: cationic polymers; colon cancer; drug targeting; rat model; sialic acid.

INTRODUCTION

Adjuvant chemotherapy is a common regimen following surgical removal of colorectal tumors. It is expected to decrease the rate of local recurrence, thus improving survival after adenocarcinomas and polyps resection (1). However, its efficacy is not unequivocal. A recent review summarized that recurrence occurs in 20–25% of Stage II patients despite surgery, whereas adjuvant chemotherapy prevents recurrence in an additional 1 to 6% of the patients (2). The efficacy of adjuvant therapy could be improved if designed to target cancer cells or malignant tissues by aiming at molecular targets (3–5). The merit of such a study would not only lead to a more competent chemotherapy, but also provide diagnostic tools.

In a previous study we found that cationic acrylamide copolymers accumulated in cancerous polyps of the rat colon more than in adjacent normal tissues (6). Furthermore, it has been reported that cationic vehicles increased the uptake of various drugs to different malignant tissues (7–10). This ability of cationic drug delivery systems to target a tumor mass is of great interest, but has of yet to be explained (11).

Sialic acid (SA) is overexpressed in colon cancer tissues (12–14). Moreover, sialyl-transferase expression was shown to be elevated in human colorectal cancer specimens, compared with the normal surrounding mucosa, and a further increase in the expression was detected in metastasis, compared with primary tumors (15).

In terms of directed drug delivery, SA can be targeted by lectins from various origins (16,17), therefore it was suggested as a mucosal tag for lectin-containing drug delivery systems. However, their potential use is restricted by competition of nutritional lectins, stability and toxicity (18). Due to the negative charge of SA in physiological conditions the use of positively charged conveyers is a much simpler attractive targeting tool.

[1] Faculty of Medicine, School of Pharmacy, Department of Pharmaceutics, The Hebrew University of Jerusalem, Jerusalem, Israel.
[2] Faculty of Medicine, School of Pharmacy, Department of Medicinal Chemistry and Natural Products, The Hebrew University of Jerusalem, Jerusalem, Israel.
[3] The Hebrew University of Jerusalem School of Pharmacy, P.O. Box 12065, Jerusalem, 91120, Israel.
[4] Affiliated with the David R. Bloom Center of Pharmacy, Jerusalem, Israel.
[5] To whom correspondence should be addressed. (e-mail: avri@cc.huji.ac.il)

ABBREVIATIONS: CA-20, cationized acrylamide copolymer containing 20% molar ratio of cationic monomer; CA-60, cationized acrylamide copolymer containing 60% molar ratio of cationic monomer; CA-100, polymerized cationic monomer; DMH, 1,2-dimethylhydrazine dihydrochloride; DTT, dithiothreitol; FITC, fluorescent isothiocyanate; WGA, wheat germ agglutinin; SA, sialic acid.

The goals of the present study were to (a) search for a possible relationship between the metastatic stage of colon carcinoma and SA expression *in vitro* (b) analyze whether SA is preferentially expressed in induced colon polyps of the rat, (c) explore the possibility of targeting cancer cells, and cancerous polyps with cationic acrylamide copolymers, tailored to contain increasing amounts of cationic modules and (d) elucidate the possible role of SA in the expected differential attachment of the cationic copolymers *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Unless stated otherwise, all materials were purchased from Sigma (St. Louis, MO, U.S.A.). Solvents were of analytical grade and water was double distilled.

Synthesis of the Cationic Monomer N-acryloyl, N'- (tert-butyl-carbonyl) Diaminoethane

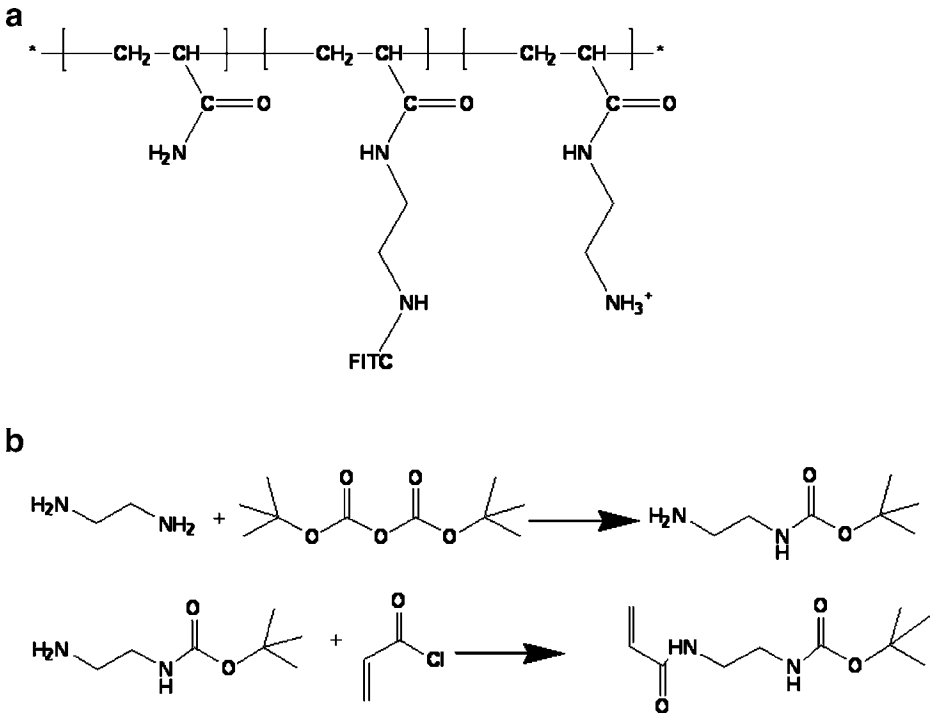
The monomer was prepared as previously described (6). Briefly, a solution of di-tert-butyldicarbonate (10 mmole), dissolved in 50 ml of dry dioxane, was added dropwise over 1 h to a solution of ethylenediamine (80 mmole) in dry dioxane (50 ml). The reaction was stirred overnight, the dioxane evaporated and water (50 ml) was added. The aqueous solution was filtered and extracted with dichloromethane, which was then dried and evaporated to produce yellowish oil. The product (1 g), chloroform (80 ml), tri-ethyl-amine (40 mmole) were mixed and cooled to 0°C. Acryloylchloride (7.5 mmole) was then added dropwise over 3 h. The chloroform phase was rinsed with water, dried, evaporated and the product was re-crystallized from ether. The resulting BOC protected cationic monomer (Scheme 1b), N-acryloyl, N'-(tert-butyl-carbonyl)-diaminoethane was characterized by LCMS, ^{1}H-NMR, ^{13}C-NMR and microanalysis. The molecular weight of the monomer was 236.2 g/mole and the yield averaged 84%.

Polymer Preparation and their Fluorescent Tagging

Three types of cationic acrylamide copolymers labeled with fluorescein isothiocyanate (FITC) were prepared: CA-20, CA-60, and CA-100 denoting the percent molar ratios of the cationic monomer in each copolymer.

Copolymerization of acrylamide (0.2 g), and the BOC protected cationic monomer was performed by heating at 90°C for 12 h in 10 ml of nitrogen-bubbled acetonitrile, using benzoylperoxide (100 ml of 50 mg/ml solution) as an initiator in sealed glass vials. After polymerization, the BOC protecting groups were removed with HCl in methanol (1 M). Polymerization and the subsequent removal of the protecting groups to produce the various CA copolymers were verified by ^{1}H-NMR. The molecular weight of the polymers was determined by GPC, employing a Spectra Physics (Darmstadt, Germany) pump, a refractive index detector and a Shodex KB-803 (Japan) column. Pullulan (PSS, Mainz, Germany) with a molecular weight range between 1,700 and 212,000 served for standard curves. The eluent was 0.05 M $NaNO_3$ in water. The nitrogen content of the polymers was determined by microanalysis and was used to calculate the charge density of the polymers, as previously described (6).

FITC (0.01 mmole, dissolved in 0.1 ml of anhydrous DMSO) was added to the polymer solutions (1 mmole, dissolved in 10 ml of 0.1 M sodium carbonate buffer, pH 9) and the reaction was incubated for 8 h in the dark, with constant stirring. The reaction was dialyzed (cut off 6000 D) against double distilled water (1:10,000) to remove unreacted fluorescein isothiocyanate (FITC). The dialyzed polymer



Scheme 1. The N-acryloyl-diaminoethane polymer tagged with FITC (**a**) and its synthesis (**b**).

solutions were then lyophilized to obtain FITC-labeled polymer powders. The general structure of the FITC labeled cationic polymers is shown in Scheme 1a. The FITC content in the polymer was determined spectrofluorometrically (at absorbance of 485 nm, emission of 528 nm) and found to be 1.03, 1.56 and 2.07% w/w for CA-100, CA-60 and CA-20, respectively. These values were used to normalize the amount of polymers in the biological studies detailed below.

Cell Lines and Culture Media

IEC-6, a non-transformed epithelial cell line from the rat small intestine; SW-480, an established cell line from human colon cancer metastatic lymph node (Dukes' stage B) and SW-620, an established cell line from human recurrent colon cancer metastatic lymph node from the same individual one year after establishment of the SW-480 (Dukes' stage C), were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (60 mg/l), and streptomycin (100 mg/l) (Biological Industries, Beit Haemek, Israel) at 37°C in a humidified atmosphere of 5% CO2 /air. The DMEM medium of the IEC-6 cells was supplemented with insulin (0.1 U/ml).

Rats, Anesthesia and Euthanasia

Rat (Sabra, 250 g) studies were conducted in accord with the Principles of Laboratory Animal Care (NIH Publication no. 85–23, 1985 Revision). The Mutual Committee for Animal Welfare of the Hadassah University Hospital and the Faculty of Medicine of The Hebrew University of Jerusalem approved the study protocol. Anesthesia was performed by an intraperitoneal injection of 100 mg/kg body weight of ketamine (Ketaset™, 0.1 g/ml Fort Dodge, USA). Euthanasia of the anesthetized rats was carried out by chest wall puncturing.

Induction of Colon Cancer in the Rats

Induction of colon neoplasia in rats was performed by a weekly subcutaneous injection of the carcinogen, 1,2-dimethylhydrazine dihydrochloride (DMH), at a dose of 4 mg/100 g rat body weight, over 5 weeks (19). After the treatment, the rats were kept for an additional 10 weeks with free access to food and water, and 12 h of alternating light/dark cycles at 22°C, and their weight was monitored weekly.

Cell Lines Interaction with the Cationic Polymers and WGA

IEC-6, SW-480 and SW-620 cell lines were grown in 96-multi-well plates to confluence. Immediately before the experiments, the growth medium was aspirated and the cells were washed with PBS to eliminate all traces of the medium. Cells were incubated for 30 min in PBS with or without neuraminidase 0.5 U/ml. Each of the two groups was divided to four subgroups and incubated in 50 μg/ml FITC-labeled WGA, CA-20, CA-40 or CA-100, for 15 min at 37°C. The cells were washed with PBS before each of the treatments and after the final incubation with the FITC labeled agents. Fluorescence of each well was quantified by Perkin-Elmer LS 50B luminescence spectrofluorometer (Norwalk, CT, USA), at 485 nm (absorbance), 528 nm (emission).

For imaging of the specific interaction between the three types of cell lines and FITC-WGA, cells were plated on glass cover-slips (18 mm diameter) in 12-multi-well plates and grown to 50–70% confluence. After a PBS rinse, the cells were incubated for 30 min in PBS with or without neuraminidase (0.5 U/ml in PBS) followed by incubation with 50 μg/ml of FITC-WGA for 15 min. Each incubation was preceded and ended by a PBS rinse. The cells were then fixed (10 min) with freshly prepared 4% formaldehyde in PBS (pH 7.4) and mounted with DABCO mounting medium. The differences between binding patterns of WGA to the cell lines were examined in a confocal microscope (Zeiss-410, Carl Zeiss AG, Germany) with 60 X plan-neofluor oil lens.

Polyps and Healthy Adjacent Epithelium Interaction with the Cationic Polymers and WGA

DMH induced rats were sacrificed and the colon was retrieved, cut open and washed with PBS. The polyps were identified (2–3 per rat) and harvested together with adjacent mucosal tissues.

Polyps and the normal adjacent epithelium were dissected, weighed and incubated in either PBS or 20 mM dithiothreitol (DTT) in PBS for 30 min at 37°C. Each of the two study groups was divided into two subgroups and incubated in either PBS or 0.25 U/ml neuraminidase for 60 min at 37°C. Each of the four sub-groups was further divided into another four subgroups that were incubated, separately, with 50 μg/ml of either of FITC-labeled wheat germ agglutinin (FITC-WGA), CA-20, CA-40, or CA-100 polymers, for 15 min at 37°C. The differences between binding patterns of the lectin and the polymers to the various tissues were examined by fluorescence detection using an Perkin-Elmer LS 50B luminescence spectrofluorometer (Norwalk, CT, USA), at 485 nm (emission), 528 nm (absorbance). Results were normalized to the tissue dry weight. Tissues were washed with fresh aliquots of PBS before each of the treatments and after the final incubation with the FITC labeled agents.

For imaging of the interaction between the polyps or the normal adjacent tissues and FITC-WGA, the various tissues were frozen with liquid nitrogen, mounted on a microtome stub with OCT embedding medium (Triangle Biomedical Sciences, Durham, NC) and sectioned with a cryostat apparatus (Leica CM 3000, Limburg, Germany) at −20°C, to obtain 10 μm sectioned specimens. The sections were incubated in 20 mM DTT in PBS for 30 min, followed by incubation in 50 μg/ml FITC-WGA for 15 min, washed, and images were taken with a confocal microscope (Zeiss-410, Carl Zeiss AG, Germany) with 60 X plan-neofluor oil lens.

Statistical Analysis

Data were analyzed by the Kruskal-Wallis test. A difference was considered to be statistically significant when the p value was < 0.05. When a difference between the groups was obtained, a Mann-Whitney test was used to analyze the significance of the difference between the means of the individual group ($p<0.05$) (computed by SPSS Sigma-Stat 3.0, Aspire Software, Leesburg VA).

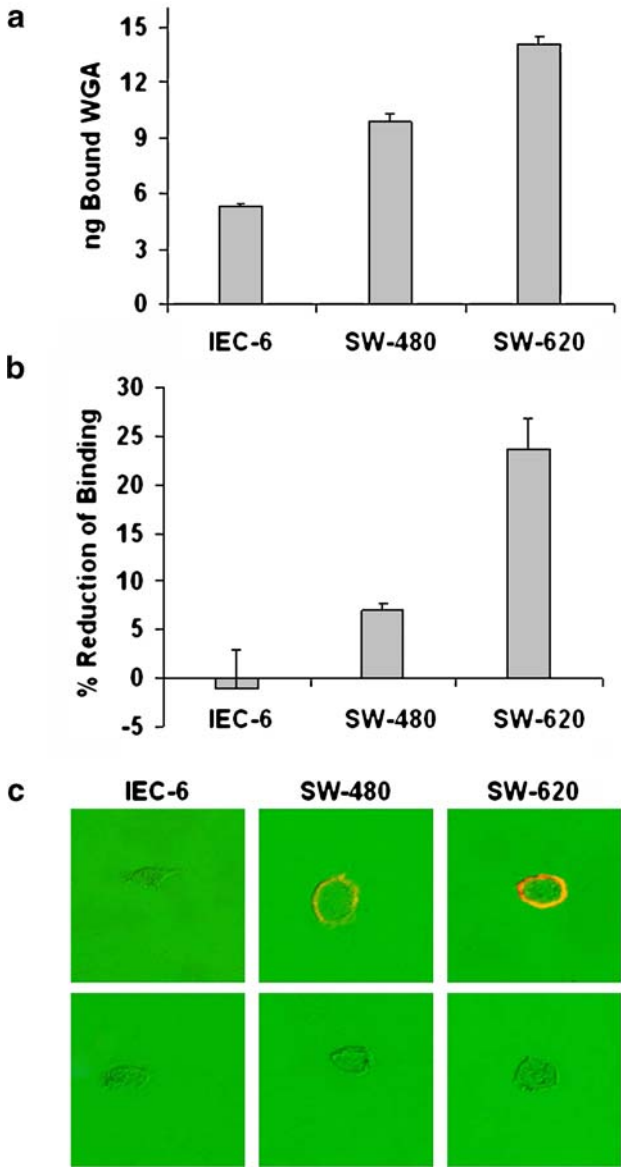


Fig. 1. The specific attachment of FITC-WGA to the two human colon cancer cell lines, SW-480 and SW-620 and the non-transformed epithelial cell line from the rat small intestinal, IEC-6. **a** Amounts of FITC-WGA bound to IEC-6, SW-480 and SW-620 cell lines. Shown are the mean values of five different experiments ± SEM. **b** The reduction in FITC-WGA binding to the three cell lines (expressed in % reduction), caused by neuraminidase. Shown are the mean values of four different experiments ± SEM. **c** Representative confocal microscopy images of FITC-WGA attached to the IEC-6 (*left*), SW-480 (*middle*) and SW-620 (*right*), with (*lower panel*) or without (*top panel*) pretreatment with neuraminidase (Magnification ×50).

RESULTS

The capability of WGA to bind to the three types of cell lines tested correlated with their metastatic stage (Fig. 1a); the lowest amount of FITC-WGA was found in the non-cancerous IEC-6 cells while the highest amount was found in the highly metastatic SW-620 cell line. Pretreatment of the different cell lines with neuraminidase reduced the binding between WGA and the different cell lines according to their stage of metastasis (Fig. 1b). The most significant reduction in the binding of WGA was found in SW-620 cell line, while no reduction was found in the binding with the IEC-6 cell line. The correlation between the metastatic stage of cell lines and the WGA binding and binding obliteration by neuraminidase was confirmed by confocal microscopy. The degree of binding was expressed as intensity of the fluorescent signal (Fig. 1c).

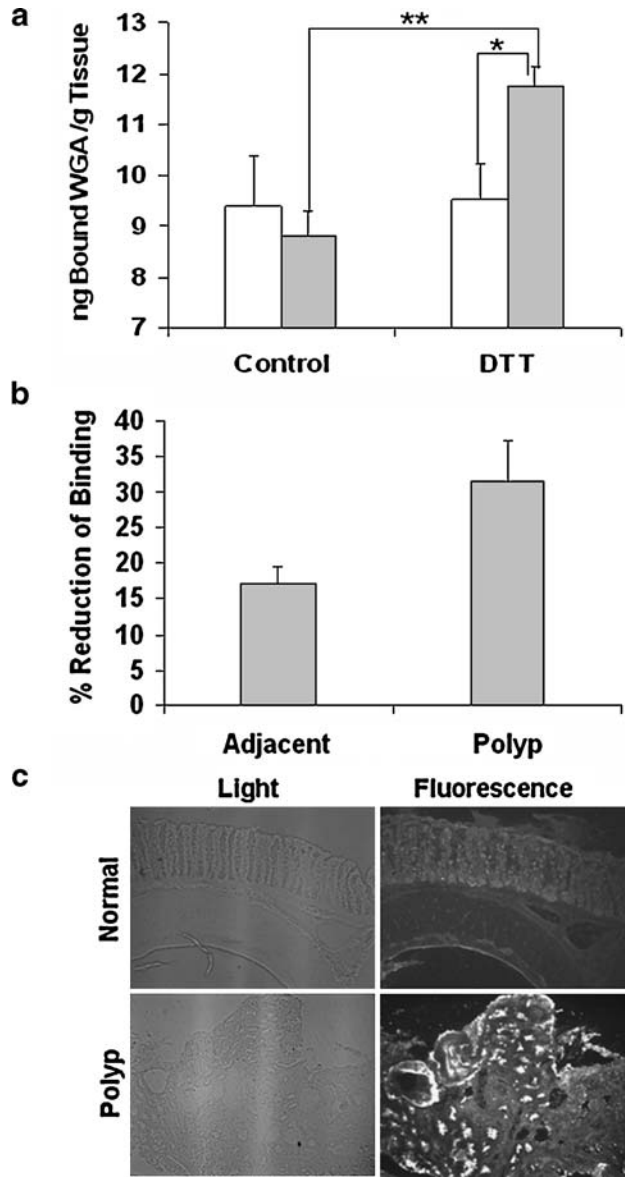


Fig. 2. Attachment of FITC-WGA to the epithelium of malignant and normal tissues of the rat colon and the involvement of mucus and SA. **a** Amounts of FITC-WGA bound to normal (*empty columns*) and malignant (*filled columns*) colonic epithelium after mucus removal with DTT (*right columns*). Shown are the mean values of four different experiments ± SEM. * $p<0.05$; ** $p<0.001$. **b** The reduction (expressed in % reduction) in FITC-WGA binding to malignant (*right*) and normal adjacent (*left*) epithelium of the rat colon, caused by neuraminidase. Shown are the mean values of four different experiments ± SEM. **c** Representative light (*left*) and confocal (*right*) microscopy images of normal (*top panel*) and malignant (*lower panel*) colonic epithelium sections after incubation with FITC-WGA (Magnification ×20).

Investigation of the WGA binding to polyps and normal colonic rat tissues revealed a non SA dependent interaction of WGA with the mucus layer covering them. Removal of the mucus layer by DTT caused differential enhancement of the binding to the polyps (Fig. 2a). Mucus removal followed by incubation with neuraminidase reduced WGA binding to the polyps more than normal tissues, expressed as percent of reduction of binding (Fig. 2b). Light microscopy showed the difference in histology of the polyp and the adjacent normal tissue (Fig. 2c, left column), while confocal microscopy illustrated the preferential binding of WGA to polyps compared to adjacent normal tissues expressed as intensity of the fluorescent signal (Fig. 2c, right column).

Three types of cationic acrylamide copolymers containing increasing molar ratios of the cationic monomer were then used to elucidate the potential role of positive charge in the attachment to the different tissues and cell lines.

The binding of the polymers to the cell lines correlated with their cationized monomer content, while dependency on metastatic stage was profound in the CA-100 polymer (highest degree of cationization) (Fig. 3a). Pretreatment with neuraminidase decreased the binding of CA-100 polymer to the cell lines. A marked decrease was observed in the binding to the malignant cell lines SW-480 and SW-620, while a much lower effect was found in the binding to the non-transformed IEC-6 cell line (Fig. 3b).

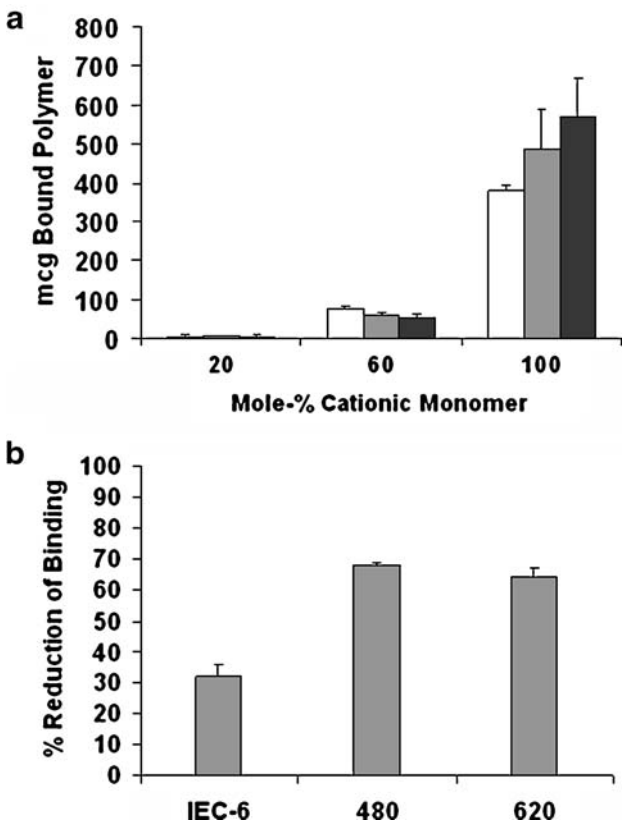


Fig. 3. The specific attachment of the cationic acrylamide copolymers to the two human colon cancer cell lines, SW-480 and SW-620 and the non-transformed epithelial cell line from the rat small intestinal, IEC-6. **a** The effect of charge density [mole-% fraction of the N-acryloyl, N'-(tert-butyl-carbonyl) diaminoethane monomer in the polymer] on polymer attachment to IEC-6 (*empty columns*), SW-480 (*grey columns*) and SW-620 (*black columns*) cell lines. Shown are the mean values of 5 different experiments ± SEM. **b** The reduction (expressed in % reduction) in the cationic polymer CA-100 (maximal charge density) binding to the three cell lines, caused by neuraminidase. Shown are the mean values of five different experiments ± SEM.

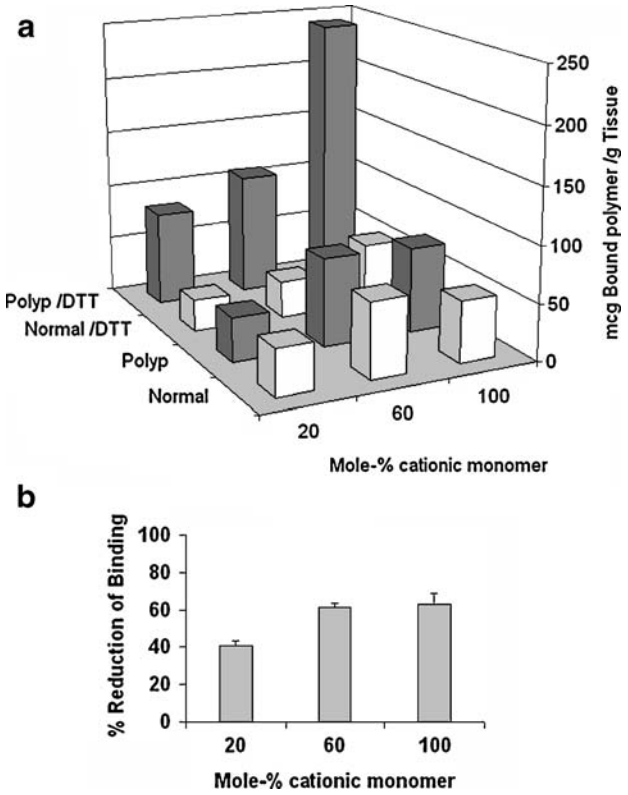


Fig. 4. The differential involvement of SA and the interference of mucus lining in the charge-dependent attachment of the three cationic polymers (CA-20, CA-60 and CA-100) to polyps and adjacent normal epithelium of the DMH induced rats. **a** The effect of mucus removal, by DTT, from the surface of polyps and normal adjacent epithelium in the rat colon, on the attachment of the three cationic polymers [expressed in mol-% content of the cationic monomer N-acryloyl-diaminoethane to the normal (*empty columns*) and malignant (*filled columns*) tissues. Shown are the mean values of four different experiments. SEM values of all bars did not exceed ±20%. **b** Reduction in the binding of the three cationic polymers to the epithelium of the rat colon polyps after pre-treatment with neuraminidase. Shown are the mean values of four different experiments ± SEM.

The adsorption of the three types of cationic acrylamide copolymers with increasing molar ratios of the cationic monomer onto the epithelium of colonic polyps of the rat and the healthy surrounding tissues is summarized in Fig. 4a. A relatively low binding of the three types of cationized copolymers was found in normal epithelia adjacent to the polyps, with or without mucus removal by DTT (Fig. 4a). On the other hand, pretreatment of the polyps with DTT significantly increased the binding of the three cationic copolymers, and the degree of binding corresponded with the ratio of the cationic monomer in the polymer (Fig. 4a). Mucus removal, followed by treatment with neuraminidase differentially reduced the binding of the copolymers with the polyps; the reduction in the binding of C-60 and C-100 was more pronounced than the reduction in the binding of C-20 (Fig. 4b).

DISCUSSION

Although surgery is the most common medica practice used to treat colorectal cancer, chemo-, radio- and biological therapies, alone or in combination, are used in adjuvant or neoadjuvant courses. Overexpression of several receptors on cancer cells (estrogen receptor, androgen receptor, CD20 or vascular epidermal growth factor receptor) have been targeted in an attempt to increase the efficacy of systemic colorectal cancer therapy (20,21).

In a previous study we targeted the intestinal epithelium in experimental colon carcinoma with cationic acrylamide copolymers. Using phenyl boronic acid as a marker, we found that the cationic polymers accumulate preferentially in DMH induced malignant polyps of the rat colon (6). These results were in agreement with previous reports, in which cationic, but not anionic or neutral, liposomes were able to target tumor vasculature(22). Recently, cationic platforms have attracted much intention due to their inherent, yet unexplained, ability to target growing tumor mass (11). In our experimental systems we found that cationic vehicles were able to accumulate in the epithelium of the colon carcinomas after luminal application, an observation which led us to the quest for the possible explanation of this specific binding and its potential implication. The hypothesis of the present study was that overexpression of the negatively charged SA on the luminal aspect of polyps in the colon of DMH induced rats, is responsible for the electrostatic binding of the cationic polymer.

The first stage of the study included the use of WGA for the detection of the possible differential expression of SA in colonic malignant and normal tissues (23). Since glucosamine residues are also known to interact with WGA, the reaction specificity was verified by pretreatment of the examined cells or tissues with neuraminidase, an enzyme known to chop off terminal SA residues from glycoproteins (24).

The malignant, human colon cancer cell lines, SW-480 and SW-620, representing increased metastatic stages (Stage II and III, respectively) and the non-transformed IEC-6 cell line representing normal epithelia were used to evaluate the metastatic stage-dependent SA expression. The binding studies showed that the magnitude of interaction of the different cell lines with WGA correlated with their metastatic stage (Fig. 1a). Pretreatment with neuraminidase did not affect WGA interaction with the normal cells, while the interaction with the malignant cell lines was reduced in accord with their metastasis stage (Fig. 1b). The binding interactions were visualized by confocal microscopy (Fig. 1c), which, likewise, showed a greater SA overexpression in the membrane of the malignant colonic cell lines compared with the normal cell line. Similar results have been reported previously by de Albuquerque Garcia Redondo *et al*. who found, using high-resolution scanning electron microscopy, significant differences in cell-surface SA expression between the IEC-6 cell line, and the colon adenocarcinoma cell lines Caco-2 and HCT-116 (25).

The second part of the study included binding analysis to whole epithelial tissues. When considering targeting epithelial SA with lectins in the gastrointestinal tract, the mucus barrier should be considered (26). We, therefore, removed the mucus lining from the colonic epithelium of the DMH treated rats by the mucolytic agent DTT (27). Before mucus removal WGA binding to the normal and malignant tissues was equal. However, after DTT treatment the magnitude of interaction between the WGA and epithelial surface of the polyp increased, while no difference could be detected in the interaction with the normal tissue (Fig. 2a). This suggests a specific interaction between the WGA and the epithelium itself rather than with the mucus coat. Treatment of the tissues with neuraminidase, after mucus removal, reduced profoundly WGA interaction with the polyps, and to a much lesser extent with the normal, surrounding tissues (Fig. 2b). Histological examination showed the typical organization of the epithelial cryptal structure of the normal tissues, in contrast to the chaotic structure of the polyps (Fig. 2c, left column). The preferential binding of WGA to the polyps after mucus removal was visualized by confocal microscopy (Fig. 2c, right column).

The findings, expressed in Figs. 1 and 2, suggest that the apical cell surface, rather than the mucus layer, of the malignant epithelium of the rat colon is characterized by overexpression of SA. This overexpression was anticipated to serve as a potential target for the cationic acrylamide copolymers used in our study.

To demonstrate this, three types of copolymers were prepared, CA-20, CA-60 and CA-100, containing elevated molar ratios of the cationic monomer N-acryloyldiaminoethane (20, 60 and 100% respectively), copolymerized with acrylamide, tagged with 1% (molar ratio) of FITC. The binding magnitude of the copolymers to the cell lines correlated with their cationic charge. The higher the positive charge density (i.e. the higher content of cationic monomer), the higher the amount of polymer that bound to all cell types (Fig. 3a), demonstrating that the interaction of the polymers with the cell lines was electrostatic in nature. Similar results have been reported by Campbell *et al*. who found, in liposomal preparations, that increasing the % molar ratio of cationic components from 10 to 50, resulted in a 100% increase in their tumor accumulation (28).

In addition to exerting the best binding properties, CA-100, the polymer with the maximal positive charge density, displayed differential binding to the cell lines. Although not significant, the higher the metastatic stage of the cells, the greater the attachment of the CA-100 was observed (Fig. 3a). As in the case of the WGA experiments, pretreatment of the cell lines with neuraminidase markedly decreased the interaction between CA-100 and the metastatic cell lines, and to a lesser extent with the IEC-6 cells (Fig. 3b), corroborating that the interaction between the polymers and the cell lines was SA dependent.

Binding studies in the DMH induced rat model showed that mucus removal from the surface of the colon epithelium did not increase the binding of all three cationic copolymers to the normal epithelium. It did, however, profoundly increase their binding to the polyps (Fig. 4a). These results support our previous finding in which the addition of mucin to the incubation medium interfered with the interaction between cationic acrylamide copolymers and polyps of the rat colon (6). In the present study, mucus removal allowed improved interaction between all three cationic copolymers and the polyps. Moreover, the magnitude of the interaction directly correlated with the cationic monomer ratio in the copolymer (Fig. 4a). Mucus removal followed by treatment with neuraminidase significantly reduced the interaction of the three polymers with polyps (Fig. 4b).

The results, as summarized in Figs. 3 and 4, clearly indicate that the interaction of the cationic polymers with either the tumor cells or the malignant tissues of the colon is of an electrostatic nature and SA dependent, thus supporting our work hypothesis and may explain the preferential accumulation of cationic carriers in malignant tissues.

It is speculated that positively charged platforms could be exploited as a targeting tool towards malignant epithelia of the colon. For example, conjugating anti-neoplastic drugs to cationic polymers could increase the specificity of adjuvant therapy after tumor resection. This, in turn, could increase the therapeutic efficacy and reduce locoregional recurrence. One should bear in mind, however, that mucus poses a physiological barrier to free access of targeted polymers to the intestinal epithelium. This problem should be taken care of when designing targeted drug carrier systems. An example for a possible solution is the surprising observation of Lai *et al*. who found that the mobility of PEGylated particles in fresh cervical mucus is increased with size (29).

The findings of this study could also be exploited for diagnostic purposes. Recent reports indicate that polyp dimensions as detected by conventional optic colonoscopy and used as criteria for polyp resection, are not necessarily associated with the malignancy state of the tumor (30,31), in particular for polyps less than 10 mm (32,33), which may lead to false negative diagnosis. An interesting application deduced from this report could be the implementation of a luminal (whether oral or rectal) pretreatment of the colon with cationic polymers, conjugated with a detectable marker to provide a preferential "tagging" of malignant tissues in this organ. Visualization could be accomplished by colonoscopy, CT or MRI to increase the resolution of currently used diagnostic procedures.

ACKNOWLEDGEMENTS

The results reported here are included in the dissertation project of A. K. Azab in partial fulfillment of his Ph.D. degree requirements at The Hebrew University of Jerusalem. The study was supported by a research grant no. 1358/05 from the Israeli Science Foundation.

REFERENCES

1. J. P. Arnoletti and K. I. Bland. Neoadjuvant and adjuvant therapy for rectal cancer. *Surg. Oncol. Clin. N. Am.* **15**:147–157 (2006).
2. A. B. Benson 3rd. New approaches to the adjuvant therapy of colon cancer. *Oncologist* **11**:973–980 (2006).
3. R. Duncan, M. J. Vicent, F. Greco, and R. I. Nicholson. Polymer-drug conjugates: towards a novel approach for the treatment of endocrine-related cancer. *Endocr. Relat. Cancer* **12**(Suppl 1):S189–S199 (2005).
4. R. K. Jain. Transport of molecules, particles and cells in solid tumors. *Ann. Rev. Biomed. Eng.* **1**:241–263 (1999).
5. A. Nori and J. Kopecek. Intracellular targeting of polymer-bound drugs for cancer chemotherapy. *Adv. Drug Deliv. Rev.* **57**:609–636 (2005).
6. A. K. Azab, M. Srebnik, V. Doviner, and A. Rubinstein. Targeting normal and neoplastic tissues in the rat jejunum and colon with boronated, cationized acrylamide copolymers. *J. Control. Release* **106**:14–25 (2005).
7. S. S. Goldman, N. D. Volkow, J. Brodie, and E. S. Flamm. Putrescine metabolism in human brain tumors. *J. Neurooncol.* **4**:23–29 (1986).
8. A. R. Holmberg, M. Wilchek, M. Marquez, J.-E. Westlin, J. Du, and S. Nilsson. Ion exchange tumor targeting: a new approach. *Clin. Cancer Res.* **5**:3056s–3058s (1999).
9. N. D. Volkow, S. S. Goldman, E. S. Flamm, H. Cravioto, A. P. Wolf, and J. Brodie. Labeled putrescine as a probe in brain tumors. *Science* **221**:673–675 (1983).
10. M. Xu, Q. R. Chen, D. Kumar, S. A. Stass, and A. J. Mixson. In vivo gene therapy with a cationic polymer markedly enhances the antitumor activity of antiangiogenic genes. *Mol. Genet. Metab.* **64**:193–197 (1998).
11. C. R. Dass, and P. F. Choong. Targeting of small molecule anticancer drugs to the tumour and its vasculature using cationic liposomes: lessons from gene therapy. *Cancer Cell Int.* **6**:17 (2006).
12. F. Dall'Olio and D. Trere. Expression of alpha 2,6-sialylated sugar chains in normal and neoplastic colon tissues. Detection by digoxigenin-conjugated Sambucus nigra agglutinin. *Eur. J. Histochem.* **37**:257–265 (1993).
13. M. J. Vierbuchen, W. Fruechtnicht, S. Brackrock, K. T. Krause, and T. J. Zienkiewicz. Quantitative lectin-histochemical and immunohistochemical studies on the occurrence of alpha(2,3)- and alpha(2,6)-linked sialic acid residues in colorectal carcinomas. Relation to clinicopathologic features. *Cancer* **76**:727–735 (1995).
14. K. Yamashita, K. Fukushima, T. Sakiyama, F. Murata, M. Kuroki, and Y. Matsuoka. Expression of Sia alpha 2->6Gal beta 1->4GlcNAc residues on sugar chains of glycoproteins including carcinoembryonic antigens in human colon adenocarcinoma: applications of Trichosanthes japonica agglutinin I for early diagnosis. *Cancer Res.* **55**:1675–1679 (1995).
15. P. Gessner, S. Riedl, A. Quentmaier, and W. Kemmner. Enhanced activity of CMP-neuAc:Gal beta 1-4GlcNAc:alpha 2,6-sialyltransferase in metastasizing human colorectal tumor tissue and serum of tumor patients. *Cancer Lett.* **75**:143–149 (1993).
16. B. P. Peters, S. Ebisu, I. J. Goldstein, and M. Flashner. Interaction of wheat germ agglutinin with sialic acid. *Biochemistry* **18**:5505–5511 (1979).
17. A. Varki. Sialic acids as ligands in recognition phenomena. *Faseb J.* **11**:248–255 (1997).
18. F. Gabor, E. Bogner, A. Weissenboeck, and M. Wirth. The lectin-cell interaction and its implications to intestinal lectin-mediated drug delivery. *Adv. Drug Deliv. Rev.* **56**:459–480 (2004).
19. J. M. Gilbert, E. M. Thompson, G. Slavin, and A. E. Kark. Chemotherapy of chemically-induced colorectal tumours. *J. R. Soc. Med.* **76**:467–472 (1983).
20. J. D. Benson, Y. N. Chen, S. A. Cornell-Kennon, M. Dorsch, S. Kim, M. Leszczyniecka, W. R. Sellers, and C. Lengauer. Validating cancer drug targets. *Nature* **441**:451–456 (2006).
21. U. Vanhoefer. Molecular mechanisms and targeting of colorectal cancer. *Semin. Oncol.* **32**:7–10 (2005).
22. C. R. Dass. Improving anti-angiogenic therapy via selective delivery of cationic liposomes to tumour vasculature. *Int. J. Pharm.* **267**:1–12 (2003).
23. M. Aubery, M. Reynier, M. Lopez, E. Ogier-Denis, J. Font, and F. Bardin. WGA binding to the surface of two autologous human melanoma cell lines: different expression of sialyl and N-acetylglucosaminyl residues. *Cell Biol. Int. Rep.* **14**:275–286 (1990).
24. M. Szeverenyi, R. Osmers, W. Rath, W. Kuhn, and L. Lampe. Changes in binding capacity of sialic acid-specific lectins in the connective tissue of the uterine cervix during its physiological maturation. *Acta Physiol. Hung.* **82**:3–13 (1994).
25. P. de Albuquerque Garcia Redondo, C. V. Nakamura, W. Souzade, and J. A. Morgado-Diaz. Differential expression of sialic acid and N-acetylgalactosamine residues on the cell surface of intestinal epithelial cells according to normal or metastatic potential. *J. Histochem. Cytochem.* **52**:629–640 (2004).
26. K. Chadee, W. A. Petri Jr, D. J. Innes, and J. I. Ravdin. Rat and human colonic mucins bind to and inhibit adherence lectin of Entamoeba histolytica. *J. Clin. Invest.* **80**:1245–1254 (1987).
27. T. A. Saldena, F. D. Saravi, O. R. Arrieta, L. M. Cincunegui, and G. E. Carra. Effect of dithiothreitol on mucus gel layer and electrophysiological properties in rat colon. *Rev. Esp. Fisiol.* **53**:385–386 (1997).

28. R. B. Campbell, D. Fukumura, E. B. Brown, L. M. Mazzola, Y. Izumi, R. K. Jain, V. P. Torchilin, and L. L. Munn. Cationic charge determines the distribution of liposomes between the vascular and extravascular compartments of tumors. *Cancer Res.* **62**:6831–6836 (2002).
29. S. K. Lai, D. E. O'Hanlon, S. Harrold, S. T. Man, Y. Y. Wang, R. Cone, and J. Hanes. Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus. *Proc. Natl. Acad. Sci. U. S. A.* **104**:1482–1487 (2007).
30. A. C. Alder, E. C. Hamilton, T. Anthony, and G. A. Sarosi Jr. Cancer risk in endoscopically unresectable colon polyps. *Am. J. Surg.* **192**:644–648 (2006).
31. M. Betes Ibanez, M. A. Munoz-Navas, J. M. Duque, R. Angos, E. Macias, J. C. Subtil, M. Herraiz, S. Rivade la, M. Delgado-Rodriguez, and M. A. Martinez-Gonzelez. Diagnostic value of distal colonic polyps for prediction of advanced proximal neoplasia in an average-risk population undergoing screening colonoscopy. *Gastrointest. Endosc.* **59**:634–641 (2004).
32. L. F. Butterly, M. P. Chase, H. Pohl, and G. S. Fiarman. Prevalence of clinically important histology in small adenomas. *Clin. Gastroenterol. Hepatol.* **4**:343–348 (2006).
33. I. C. Lawrance, C. Sherrington, and K. Murray. Poor correlation between clinical impression, the small colonic polyp and their neoplastic risk. *J. Gastroenterol. Hepatol.* **21**:563–568 (2006).