The Role of Galactose, Lactose, and Galactose Valency in the Biorecognition of *N*-(2-Hydroxypropyl)Methacrylamide Copolymers by Human Colon Adenocarcinoma Cells

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Purpose. To examine the β-galactoside and β-lactoside binding capacity of three human colon-adenocarcinoma cell lines and their sugar specificity, using *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugates of galactosamine, lactose, and triantennary galactose.

Methods. Three types of HPMA copolymers containing the saccharide epitopes galactosamine (P-Gal), lactose (P-Lac), or triantennary galactose (P-TriGal) were synthesized. The relationship between the content of the saccharide moieties, the valency of the galactose residues, and their biorecognition by the cell lines (Colo-205, SW-480, and SW-620) was investigated using flow cytometry and confocal fluorescence microscopy analysis.

Results. The binding of the glycoconjugates to the human colon-adenocarcinoma cell lines was dependent on the type and the number of bound sugar residues per macromolecule. The higher the sugar contents in the HPMA copolymers, the higher the extent of binding. Although introduction of galactoside residues into the HPMA copolymer resulted in a significant increase in the binding of the copolymers to the cells, low biorecognition of the lactoside-containing HPMA copolymers by all cell lines used was observed. The trivalent galactoside-containing HPMA copolymers did not yield a notable glycoside cluster effect for the β-galactoside-binding lectin expressed on human colon-adenocarcinoma cells. Among the various cell lines, little differences in the extent of binding of the glycopolymers to the cells were observed. The data on the internalization of HPMA copolymer conjugates obtained by confocal fluorescence microscopy correlated well with the flow cytometry analysis of their biorecognition by target cells.

Conclusions. The lectin-mediated endocytosis of the HPMA-glycoconjugates in human colon cancer cell lines suggests their potential use as targeting tools of cytotoxic drugs to colon adenocarcinoma.

KEY WORDS: HPMA copolymer; biorecognition; galectin; colon-adenocarcinoma cells; lectin-mediated drug targeting; confocal microscopy.

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INTRODUCTION

Membrane lectins on both normal and malignant cells are involved in various biologic functions, acting as specific receptors and mediating endocytosis of specific glycoconjugates (1,2). Carbohydrate ligands may provide an efficient route for shuttling small drug molecules, proteins, macromolecules, oligonucleotides, or even genes into cells (3). Because lectins recognize and bind to the terminal sugar residues of oligosaccharides, carbohydrate ligands offer significant targeting potential (4–6). The attachment of appropriate saccharides to carrier systems is being explored for promoting lectin binding at target sites.

Lectins are displayed at the surface of numerous types of tumor cells, including human colon-cancer cells (7,8). The expression of galectin-3, a β-galactoside-binding protein, has been found to correlate with tumor progression in colon cancer (9–11), and its expression in primary colon cancers is associated with advanced tumor stage and decreased survival. In fact, clinical data revealed that distant metastases expressed higher levels of galectin-3 compared with the primary tumors from which they arose. Irimura *et al.* (13) found increased levels of the 31-kDa galectin-3 protein in homogenates of advanced stage (Dukes' D) colorectal cancers compared with specimens of early-stage disease. These findings suggest a potential role for galectins in general, and for galectin-3 in particular, in colon cancer metastasis. Furthermore, the level of the lactose-binding lectins in primary tumors of patients having distant metastasis (Dukes' Stage D) was significantly higher than levels in tumors of patients without detectable metastasis (Dukes' Stages B1and B2) (12,13). These differences in endogenous lectin expression can be exploited to target drug conjugates to the surface of cancer cells with metastatic potential and to create a local increase of drug concentration in the vicinity of the tumor mass.

Considerable effort has been devoted to the development of lectin-based drug delivery systems that can be recognized specifically by endogenous lectins expressed on human colon adenocarcinoma cells as an initial step towards the improvement of clinical management of human colon adenocarcinoma. Antitumor drugs have been linked to glycoproteins, neoglycoproteins, and glycosylated polymers to carry toxic drugs specifically to human colon adenocarcinoma cells (14,15). Indeed, Gabius *et al.* (14) have shown that human colon adenocarcinoma cells primarily bind and internalize neoglycoproteins bearing either α-galactose, α-glucose, or *N*-acetyl β-glucosamine. The efficiency of neoglycoproteins to ferry 5-fluorodeoxyuridine to target cells correlated with the saccharide binding capacity of these cells (14). Putnam (15) has suggested that the biorecognition of HPMA copolymer conjugates containing 5-fluorouracil, attached covalently via oligopeptide side chains, could be exploited for the specific treatment of colon cancer.

A major constraint associated with using carbohydrates to target membrane lectins is their low binding affinity. Membrane lectins usually recognize specific complex oligosaccharides with a high affinity and recognize single sugars with low affinity (16). To compensate for the weak binding interactions, multiple carbohydrate residues may be introduced into one macromolecule to enhance its binding affinity (17). Macromolecular carriers containing multiple carbohydrate recog-

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nition moieties enhance binding affinity through cooperative polyvalent interactions. Indeed, poly-L-lysine substituted with mannose and gluconoyl residues displays a high specificity toward membrane lectins (18). The affinity of the asialoglycoprotein receptor (ASGP-R) for HPMA copolymers bearing terminal *N*-acylated galactosamine corresponded with the prevalence of this saccharide-binding ligand (19).

HPMA copolymers and their drug conjugates have been frequently studied for their potential use as anticancer drug carriers (reviewed in Ref. 20). HPMA copolymer-anticancer drug conjugates have been found to be active against numerous cancer models when introduced parenterally. Clinical data indicate the potential application of these drug delivery systems for cancer treatment (21).

In a previous study, we reported on targetable delivery systems based on HPMA copolymer conjugates containing multivalent pendant saccharide moieties. The biorecognition of fluorescein isothiocyanate (FITC)-labeled HPMA copolymers containing the saccharide epitopes galactose, lactose, or trivalent galactoside by hepatocytes at cellular and subcellular levels was investigated (22). We have demonstrated that the extent of binding increased with the increase in sugar content in the copolymer. The extent of binding was dependent on the type of sugar moiety bound to the copolymer. The most efficient recognition systems were those containing trivalent-galactoside or lactoside, probably due to their ability to target the ASGP-R on hepatocytes.

This paper reports the biorecognition and internalization of FITC-labeled-HPMA copolymer conjugates containing galactosamine, lactose, or trivalent galactose as targeting units in three human colon adenocarcinoma lines. We hypothesized that binding capacity of sugar polymers to colon cancer cells would change with the progression of metastasis. For that purpose, three human colon adenocarcinoma lines, at different stages of neoplastic progression, were selected as representative models: Colo-205 cells, established from the ascites fluids of patient with colon adenocarcinoma; SW- 480 cells, established from a primary colon adenocarcinoma; and SW-620 cells, a lymph node metastasis after tumor recurrence in the same patient. The effect of the type, contents and construct of the conjugated saccharide groups on cell recognition and internalization of the HPMA copolymers by the colon adenocarcinoma cell lines was explored to identify those saccharide moieties with best targeting properties. Specifically, a cluster construct of galactose was investigated in the context of improved affinity to the cells.

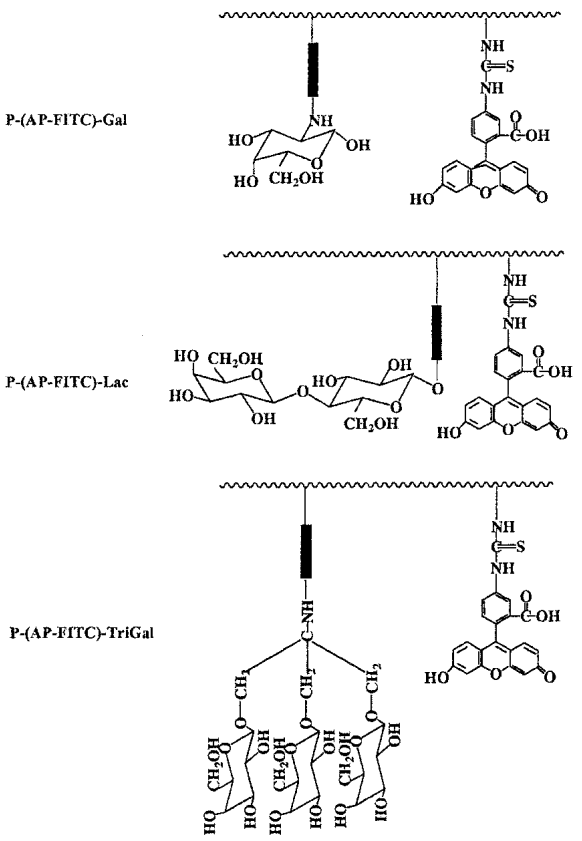

Fig. 1. Structure of the saccharide-containing HPMA copolymers. P-Gal, galactosamine-containing HPMA copolymer; P-Lac, lactose-containing HPMA copolymer; P-TriGal, triantennary galactose-containing HPMA copolymer.

MATERIALS AND METHODS

Conjugate Synthesis

The fluorescein-labeled polymer glycoconjugates were synthesized by radical precipitation copolymerization of HPMA, MA-AP-FITC, and the saccharide-containing monomers, as previously described (22). The polymers are of the general structure shown in Fig. 1. Briefly, all polymerizations were conducted using free radical copolymerization of three monomers: HPMA, MA-AP-FITC, and the saccharide-containing monomer [either MA-GG-GalN, MA-Ph-Lac, or MA-AHT-$(Gal)_3$], at 50°C for 40 h. Three copolymers of each saccharide moiety (i.e., galactosamine, lactose, or trivalent-galactose) were synthesized. The monomer feed concentrations were 3 mol% of MA-AP-FITC; 10, 20, and 30 mol% of the saccharide-containing monomer; and 87, 77, and 67 mol% of HPMA, respectively. The concentration of monomers, with respect to initiator and solvent, was in the weight ratio of 12.5:1.0:86.5, respectively. The monomer mixture of the control copolymer (P-F) did not contain carbohydrate moieties and was comprised of 98 mol% of HPMA. The polymerization was initiated using AIBN.

Synthesis of P-(AP-FITC)-GalN (P-Gal)

Galactose-containing HPMA copolymers were synthesized by copolymerization of HPMA, MA-GG-GalN, and MA-AP-FITC in acetone:dimethyl sulfoxide (DMSO) (7:3). All monomer mixtures contained 3 mol% (17.7 mg) of MA-AP-FITC; the contents of MA-GG-GalN were 10 mol% (40 mg), 20 mol% (80 mg), and 30 mol% (120mg); and of HPMA were 87 mol% (138 mg), 77 mol% (122.2 mg), and 67 mol% (106.4 mg), respectively. MA-GG-GalN and MA-AP-FITC were dissolved in DMSO, and HPMA and AIBN were dissolved in acetone. The solutions were mixed, transferred to an ampoule, bubbled for 10 min with nitrogen, sealed, and the mixture was polymerized in a water bath at 50°C for 40 h while stirring. Subsequent to polymerization, the ampoules were cooled to room temperature, opened, and the volume was reduced under vacuum. The viscous liquid thus obtained was precipitated into a large excess of acetone. The precipi-

tated polymer was dissolved in distilled water, dialyzed against water for 24 h, and lyophilized. The copolymers were denoted 10% P-Gal, 20% P-Gal, and 30% P-Gal, reflecting the amount of MA-GG-GalN in the monomer mixture. The yields were: 10% P-Gal, 140 mg (71%); 20% P-Gal, 150 mg (68%); and 30% P-Gal, 160 mg (66%). The structure of the polymers is shown in Fig. 1. The results of their characterization and methods used are listed in Table I.

Synthesis of P-(AP-FITC)-Lac (P-Lac)

The lactose-containing HPMA copolymers were synthesized by the same procedure as described for the galactosamine-containing copolymers. The feed weights were: 3 mol% of MA-AP-FITC (9.5 mg); 10 mol% (30 mg), 20 mol% (60 mg), and 30 mol% (90mg) of MA-Ph-Lac, and 87 mol% (74.6 mg), 77 mol% (66.0 mg), and 67 mol% (57.5 mg) of HPMA, respectively. The solvent system was MeOH. Subsequent to polymerization, volume was reduced under vacuum. The crude compound thus obtained was dissolved in water and the high-molecular-weight copolymer was purified on Sephadex G-25M (Amersham Pharmacia Biotech, Piscataway, NJ). The copolymers are denoted as 10% P-Lac, 20% P-Lac, and 30% P-Lac, reflecting the amount of MA-Ph-Lac in the monomer mixture. The yields were: 10% P-Lac, 90 mg (79%); 20% P-Lac, 90 mg (67%); and 30% P-Lac 120 mg (76%). The structure of polymers and the results of their characterization are shown in Fig. 1 and Table I.

Synthesis of P-(AP-FITC)-TriGal (P-TriGal)

The triantennary galactose-containing HPMA copolymers were synthesized by the same procedure as described for the galactosamine-containing copolymers. The feed weights were: 3 mol% of MA-AP-FITC (4.1 mg), 10 mol% (20 mg), 20 mol% (40 mg), and 30 mol% (60 mg) of MA-AHT-$(Gal)_3$, and 87 mol% (32 mg), 77 mol% (28.3 mg), and 67 mol% (25.0 mg) of HPMA, respectively. The solvent system was MeOH. Subsequent to polymerization, volume was reduced under vacuum. The crude compound thus obtained was dissolved in water and the high-molecular-weight copolymer was purified on Sephadex G-25M (Amersham Pharmacia Biotech). The copolymers are denoted as 10% P-TriGal, 20% P-TriGal, and 30% P-TriGal, reflecting the amount of MA-AHT-$(Gal)_3$ in the monomer mixture. The yields were: 10% P-TriGal, 30 mg (53%); 20% P-TriGal, 40 mg (55%); and 30% P-TriGal, 40 mg (45%). The structure of polymers and the results of their characterization are shown in Fig. 1 and Table I.

Control Fluorescein-Labeled HPMA Copolymer (P-F)

A fluorescein-labeled polymer conjugate without any carbohydrate moiety was synthesized as a control copolymer. The procedure used was the same as that used for the galactosamine-containing copolymer. The feed weights were 98 mol % of HPMA (320 mg) and 2 mol % of MA-AP-FITC (24 mg). The solvent system was acetone. The yields were 97% (271 mg). The characterization of copolymer is shown in Table I.

Human colon-adenocarcinoma cell lines Colo-205, SW-480, and SW-620 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). L-15 Leibovitz medium, RPMI-1640 medium, and Dulbecco's phosphate-buffered saline (DPDS) were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was obtained from HyClone (Ogden, UT). SW-480 and SW-620 cells were cultured in L-15 Leibovitz medium. Colo-205 cells were cultured in RPMI-1640 medium. Both media were supplemented with 10% FBS. Cell were grown at 37°C in a humidified atmosphere of 5% CO_2 (v/v) in air. All cells used in this study were between passages 18 and 39.

Flow Cytometric Analysis

Cell suspensions of each cell line were obtained after trypsinization of cell monolayers. Cells (1×10^6) were seeded in 24-well plates in 2 ml of the appropriate medium supplemented with 10% FBS. The cells were incubated with 10

Table I. Characteristics of the Copolymer Conjugates Containing the Three Types of the Pendant Saccharide Moieties

Polymer	Saccharide	Mol % sugar[a]	Mol % FITC[b]	Approximate molecular weight of conjugate (Daltons)[c]
10% P-Gal	GalN	14.8	2.04	15,000
20% P-Gal	GalN	20.4	2.42	16,000
30% P-Gal	GalN	29.1	2.64	16,000
10% P-Lac	Lactose	11.1	1.58	18,000
20% P-Lac	Lactose	19.9	1.76	20,000
30% P-Lac	Lactose	27.6	1.90	19,500
10% P-TriGal	Tri-Galactose	8.47	2.23	14,500
20% P-TriGal	Tri-Galactose	13.6	2.34	14,000
30% P-TriGal	Tri-Galactose	24.4	2.52	14,000
P-F	–	–	2.51	20,000

[a] The contents of galactose and lactose residues were determined spectrophotometrically using the phenol-sulphuric acid assay (22); the content of galactosamine was determined by the Morgan-Elson assay (22).

[b] The content of FITC was determined spectrophotometrically using $\epsilon = 82{,}000\ M^{-1}\ cm^{-1}$ (FITC; 495 nm, pH 9.1 (0.1 M sodium borate).

[c] Weight average molecular weights of copolymers were estimated by size-exclusion chromatography using a Superose 12 column, FLPC system, phosphate-buffered saline (PBS) buffer, pH 7.3, calibrated with poly(HPMA) fractions.

Table II. Quick Identification Reference for the Compounds Abbreviations

Abbreviation	Compound identification
HPMA	*N*-(2-hydroxypropyl)methacrylamide
P	HPMA copolymer backbone
MA-GG-GalN	*N*-methacryloylglycylglycyl-galactosamine
MA-Ph-Lac	4-Methacryloylamidophenyl-β-lactoside
MA-AHT-$(Gal)_3$	*N*-methacryloylaminohexyl-trisgalactoside
MA-AP-FITC	5-[3-(methacryloylaminopropyl)thioureidyl] fluorescein
P-Gal	FITC-labeled galactosamine-containing HPMA copolymer
P-Lac	FITC-labeled lactose-containing HPMA copolymer
P-TriGal	FITC-labeled triantennary galactose-containing HPMA copolymer
P-F	FITC-labeled HPMA copolymer (control copolymer)

μg/ml of the saccharide-containing HPMA copolymers (P-Gal, P-Lac, and P-TriGal) for 24 h at 37°C under 5% CO_2 atmosphere. The copolymer concentration and the incubation period were chosen based on preliminary optimization studies. Control cells were incubated with the same concentration of the FITC-labeled HPMA copolymer without sugar moiety (P-F). Inhibition experiments were performed with the same concentrations of the saccharide-containing copolymers, but in the presence of 150 mM free lactose in the incubation medium. After incubation, the polymer solutions were removed from the well and the cells were washed twice with 1 ml of cold phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.5% bovine serum albumin (BSA). Cells were harvested by trypsinization with 0.25% (w/v) trypsin, and were further incubated for 30 min at 4°C in PBS containing 50 mM monensin. Cell suspensions were immediately analyzed by flow cytometry using a fluorescence-activated cell sorter (FACS; Becton Dickinson, Mountain View, CA). Cells were gated according to light scattering parameters.

Confocal Microscopy

The internalization of the FITC-labeled saccharide-containing HPMA copolymer conjugates (P-Gal, P-Lac, and P-TriGal) in SW-480 cells was monitored using fluorescence microscopy. After trypsinization, 2×10^4 cells were seeded on an N1 cover slip (Corning, Corning, NY) and were cultured for 24 h. The culture medium was replaced with medium containing polymer conjugates (50 μg/ml) and was cultured for 24 h at 37°C. Unbound polymer conjugates were removed by washing the cell layer three times with cold PBS. Cells were fixed with 3% paraformaldehyde for 10 min at room temperature and were washed again. The fate of polymer conjugates in the cells was analyzed on a Bio-Rad (Hercules, CA) MRC-600 laser scanning confocal imaging system with a Zeiss (Oberkochen, Germany) Axioplan microscope with a plan-apo objective (60×, NA = 1.4, oil) and a krypton/argon laser. Images were acquired with a Fluor filter block (excitation at 488 nm, emission collected with a 515-nm barrier filter). Autofluorescence background was ascertained using control (untreated) cells. Fluorescent images were scaled to 256 gray levels. Quantitative analysis was performed using Image-Pro Plus 4.0 (Media Cybernetics, Silver Spring, MD). Fluorescence intensities were expressed in arbitrary units per square micron. Data were normalized to the number of cells per unit field.

Colocalization Study

Lysotracker Red DND-99 (Molecular Probes, Leiden, The Netherlands) was selected to visualize lysosomes in SW480 cells. Subcellular trafficking of the 20% P-TriGal product to lysosomes was implied by colocalization with the Lysotracker. SW480 cells were incubated for 24 h (5×10^4/200 μl) in the presence of 50 μg/ml polymer, or in its absence for the control. Cells were subsequently rinsed three times with media and were exposed to the Lysotracker (75 nM, 30 min, 37°C) after which they were rinsed three times with cold PBS, fixed in 3% paraformaldehyde, and mounted in Mowiol-DABCO (Aldrich Chemical Co., Milwaukee, WI; Sigma, St. Louis, MO, respectively) mounting medium. Images were acquired as above followed by a red filter analysis (excitation at 543 nm, emission collected with a 570-nm barrier filter).

Statistics

The obtained binding intensity data were analyzed using Kruskal-Wallis nonparametric analysis of variance (ANOVA) test. The number of studies was six for each experiment. The difference between variants was considered significant if $p < 0.05$.

RESULTS

The bindings of the fluorescein-labeled glycoconjugates (10 μg/ml) to the three human colon adenocarcinoma cell lines are shown in Figs. 2 and 3. At 37°C, the fluorescence intensity of all cells incubated with the glycoconjugates was greater than that of the cells incubated at 4°C, suggesting that endocytotic process was involved (Fig. 2). At 4°C, endocytosis is slowed to nearly an insignificant extent (5). At 37°C, the cells are able to endocytose the content of the extracellular space, thus trapping the glycoconjugates within the cells, resulting in a greater cell associated fluorescence. The cell-associated fluorescence of the colon adenocarcinoma cells after incubation with the saccharide-containing HPMA copolymer conjugates is summarized in Fig. 3, A through C. The flow cytometry analysis revealed strong binding of the synthesized copolymers to the cells after 24 h of incubation at 37°C. The fluorescence intensity indicates that the biorecognition of the saccharide-containing HPMA copolymers by the cells was dependent on the type and the content of the pendant saccharide moiety in the copolymers. The increased glycosylation of the copolymers with galactose and lactose correlated well with an increased cell binding. In general, no

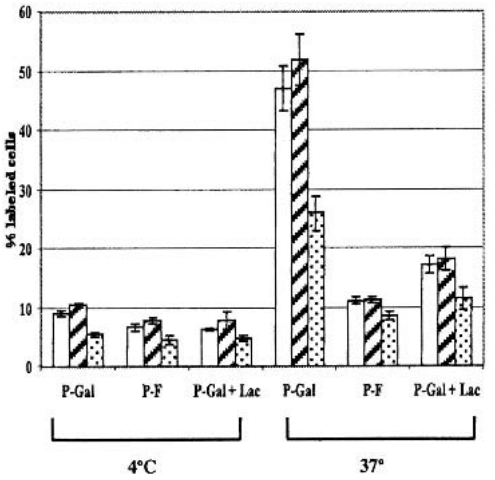

Fig. 2. Binding of the 10% P-Gal (10 μg/ml) to three human colon adenocarcinoma cells in the presence and absence of 150 mM lactose, as analyzed after 24 h of incubation at 4°C or 37°C by flow cytometry. HPMA copolymer (P-F) without any sugar moiety served as control. Colo-205, empty columns; SW-480, cross-hatched columns; SW-620, dotted columns. Six binding studies were performed for each set of experiments.

significant differences in the binding intensities between the trivalent galactoside and the galactoside-containing copolymers were observed. Their binding to all three adenocarcinoma cells was significantly higher when compared with the lactoside-containing copolymers and the control (P-F) copolymer (Fig. 3). The binding specificity was verified by inhibition studies in which it was found that 150 mM lactose was able to significantly reduce the copolymers' binding to the cells, indicating that carbohydrate-binding sites mediated the binding. Control polymers (without sugar, denoted as P-F) were barely bounded to the cells, ruling out nonspecific interaction (Fig. 3, A–C, left bars, denoted as 1). There was little difference in the glycoconjugate binding among the different cell lines that were derived from a primary tumor (SW-480) or from a metastatic lesion of the same patient (SW-620) or from metastasis in the ascites fluids of patient with colon adenocarcinoma (Colo-205). Although the differences were small, the binding intensity of the copolymers to the SW-480 cells and Colo-205 cells was 2-fold higher than the binding to the SW-620 cells.

The uptake and localization of the FITC-labeled HPMA copolymer conjugates by cells was followed by confocal fluorescence microscopy. Cells were incubated with the HPMA copolymer conjugates for 24 h at 37°C before optical sections were taken. Confocal images indicated the lysosomotropism of the conjugates, i.e., internalized glycoconjugates were transported into the lysosomal compartment of the cells. The HPMA-FITC-conjugates were accumulated in round-shaped organelles localized in the perinuclear region of cells. The internalization extent of conjugates was dependent on the type and the content of the pendant carbohydrate moiety in the copolymers. Increasing the mol % of each sugar moiety in the copolymer increased the image brightness (Fig. 4). Quantitative analysis of the fluorescence intensities of the polymers internalized into SW-480 cells (Fig. 5), expressed in arbitrary units per square micron, correlated well with the fluorescence intensity of the internalized polymers, as determined by flow

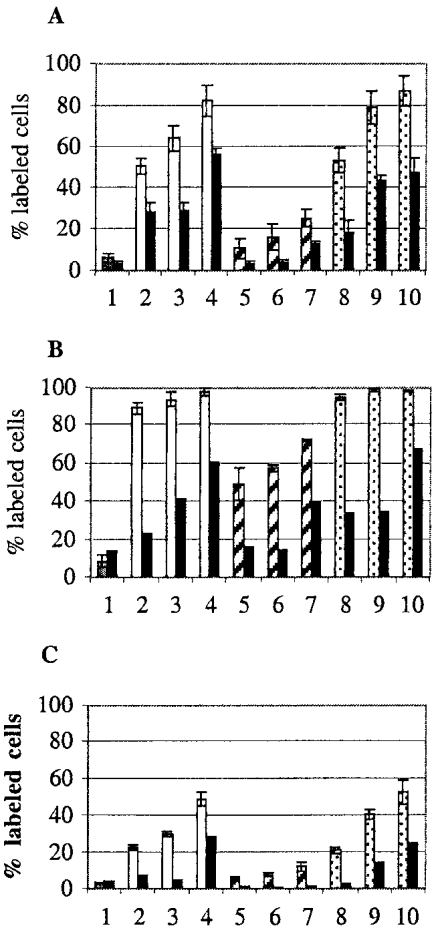

Fig. 3. Binding of the saccharide-containing HPMA copolymers (10 μg/ml) to Colo-205 (A), SW-480 (B), and SW-620 (C) human colon adenocarcinoma cells, as analyzed by flow cytometry after 24 h of incubation at 37°C, in the presence (150 mM, solid columns) or absence of lactose. 1, Nonsaccharidic control polymer (P-F); 2, P-Gal 10%; 3, P-Gal 20%; 4, P-Gal 30%; 5, P-Lac 10%; 6, P-Lac 20%; 7, P-Lac 30%; 8, P-TriGal 10%; 9, P-TriGal 20%; 10, P-TriGal 30%; gray columns (at the left of plots A, B, and C), P-F without lactose; white columns, P-Gal without lactose; cross-hatched columns, P-Lac without lactose; dotted columns, P-TriGal without lactose. Shown are the mean values of six studies ± SD.

cytometry (Fig. 3B). The trivalent galactoside and the galactoside-containing copolymers were internalized more efficiently than the lactoside-containing copolymers. The fluorescence images of cells after incubation with the nontargetable copolymer P-F were dimmer compared with the targetable polymer at the same polymer concentration, indicating that the nontargetable copolymers were internalized less efficiently.

Colocalization analysis, performed with the 20% P-TriGal copolymers, verified that the polymers were localized in the lysosomal compartments of the cell after internalization. SW-480 cells, stained with Lysotracker Red DND-99, showed organelles with similar morphology and localization to those containing the polymer (Fig. 6).

DISCUSSION

The combination of receptor-binding ligands with synthetic water-soluble polymeric conjugates may yield new tar-

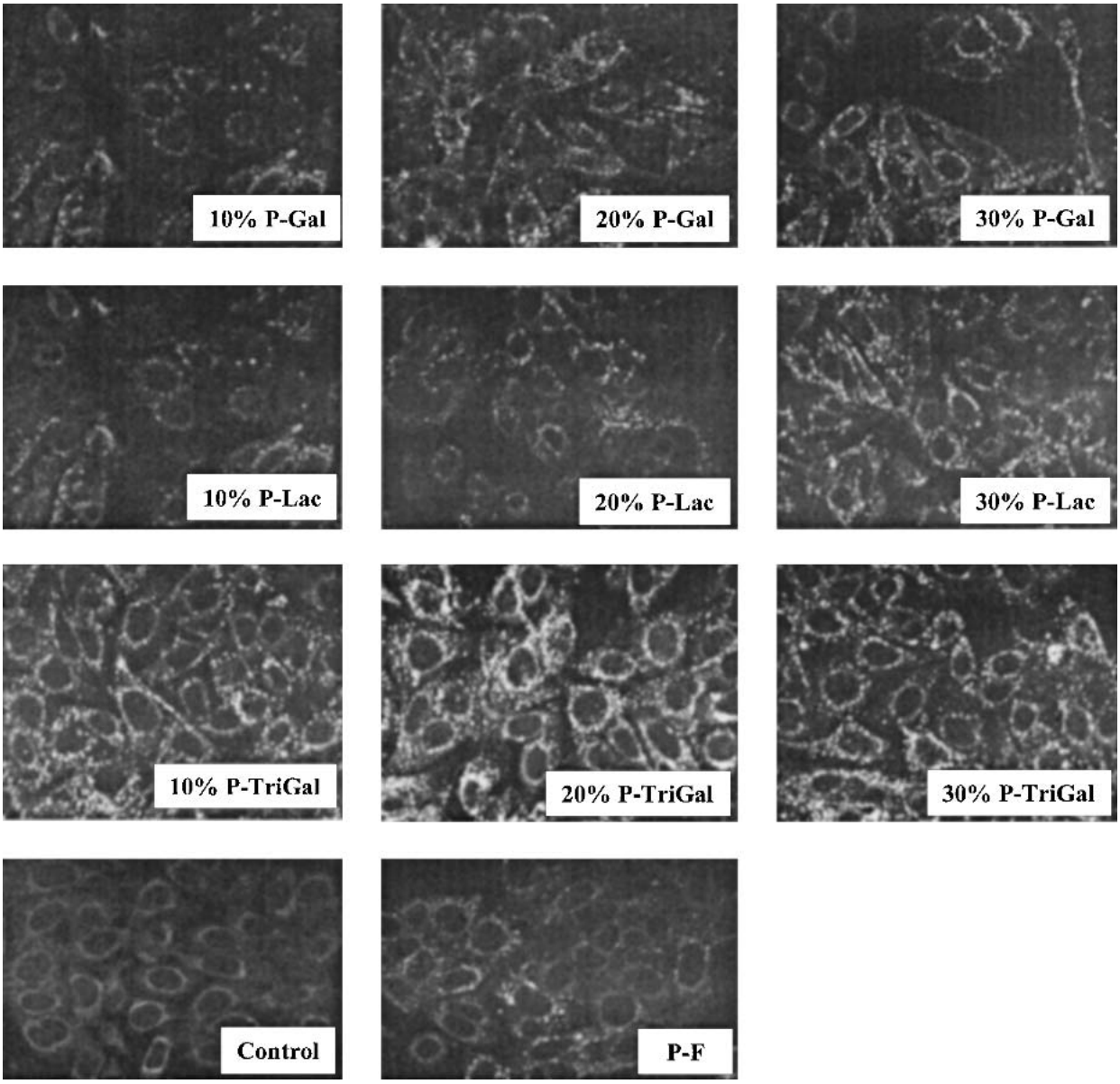

Fig. 4. Confocal fluorescence images of SW-480 cells incubated with the various saccharide-containing HPMA copolymer-FITC conjugates, as observed after 24 h of incubation at 37°C. Upper panel (left to right): 10% P-Gal, 20% P-Gal, and 30% P-Gal; second panel (left to right): 10% P-Lac, 20% P-Lac, and 30% P-Lac; third panel (left to right): 10% P-TriGal, 20% P-TriGal, and 30% P-TriGal; bottom panel (left to right): control study (cells prior to incubation); nonsaccharidic control polymer (P-F).

getable drug carriers of anticancer drugs. Many naturally occurring glycoproteins contain branched or multiantennary (cluster) structures (reviewed in Ref. 2). The advantage of incorporating numerous biorecognizable moieties into a polymeric chain is the ability to enhance its cellular uptake by enhancing adherence to the plasma membrane being internalized (23). Multivalent interactions can collectively be much stronger than the corresponding monovalent interactions (16–18). When compared with neoglycoproteins, the advantage of synthetic glycopolymers is that they can be tailored to have both lower molecular weight and high sugar density.

In a previous study, we demonstrated a stronger binding of the trivalent galactoside and lactoside-containing HPMA copolymers to hepatocarcinoma cells compared with the monovalent galactoside-containing copolymers. This biorecognition was assumed to be mediated by the ASGP-R on hepatocytes (22). In the present study, we verified that three human colon adenocarcinoma cell lines used display β-galactoside-binding capacity.

The interaction between membrane lectins, expressed on human colon adenocarcinoma cells, and multivalent ligands has not been adequately investigated. Using flow cytofluorometric methods, Gabius *et al.* (14) have previously shown that human colon adenocarcinoma cells bind and internalize neoglycoproteins bearing α-galactose, α-glucose, or *N*-acetyl β-glucosamine. However, the targeting efficiency of these conjugates was low. It was speculated that glycoconjugates containing multiple carbohydrate complexes that are tailormade to match the specificity of target lectins would improve the targeting efficiency. Recently, Andre *et al.* (24) have stud-

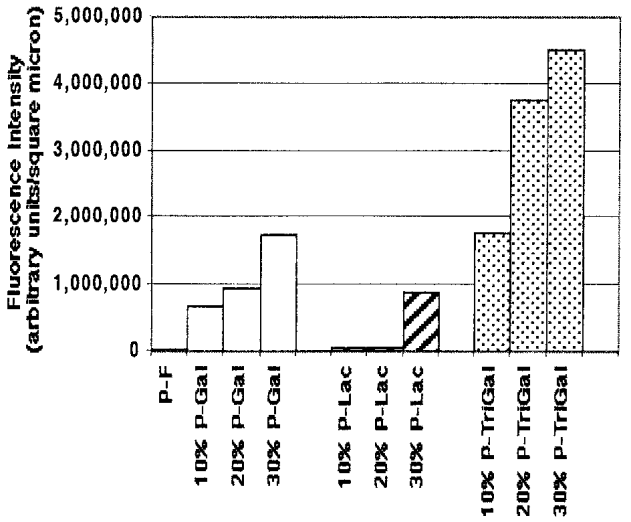

Fig. 5. Quantification of the fluorescence intensity (expressed in arbitrary units per square micron) of the confocal fluorescence images of the SW-480 cells after incubation with the various saccharide-containing HPMA copolymer-FITC conjugates and the control polymer (P-F) after 24 h at 37°C.

ied the distinct structural features of cluster glycoside ligands presenting galactose/lactose and their ability to differentiate between ASGP-R and galectins. The binding extent of labeled sugar receptors to matrix-immobilized neoglycoproteins comprised of mono-, bi-, and trivalent glycosides and to SW-480 cells was evaluated. It was found that bi- and trivalent glycosides do not exhibit glycoside cluster effect for galectin-1 and galectin-3. The cluster design of galactose and lactose residues did not improve the binding affinity.

To learn more about the binding properties of carrier systems containing multi- and multivalent carbohydrate side chains to endogenous lectins expressed on human colon adenocarcinoma cells, synthetic HPMA copolymers were designed to contain mono- and trivalent galactoside, and lactoside ligands. HPMA copolymers are water-soluble macromolecules with low immunogenicity that have been frequently used as anticancer drug carriers (20). Phase I clinical trial data seem to indicate their great potential for applications in oncology (21,25). As yet, only monosaccharides were used to target HPMA copolymers to the cell membrane of cancer cell lines (26,27). It has been previously found that HPMA copolymers containing side chains terminated in *N*-acylated galactosamine behaved similarly to the ASGP and were recognized *in vivo*. Their biorecognition by the ASGP-R of hepatocytes was dependent on the amount of bound ligand (19). It should be mentioned that synthetic macromolecules based on HPMA copolymers are flexible in an aqueous environment (28). Consequently, they could be arranged in such a conformation that several sugar moieties from a single macromolecule interact with one receptor. Therefore, better biorecognition could be achieved by increasing the concentration of the biorecognizable sugar moieties along the polymeric chain.

In this study, the designed HPMA copolymers containing multi- and multivalent carbohydrate side chains were tested to determine whether and to what extent they could improve the binding properties of the carrier system to colon cancer cells. Flow cytometry analysis and confocal microscopy demonstrated the binding and endocytosis of the glycoconjugates to three human colon-adenocarcinoma cell lines. The flow cytometry results demonstrate that the relative extent of binding of the glycoconjugates was dependent on the type and the content of the sugar moiety. The confocal microscopy results as shown in Figs. 4 and 6 demonstrated that endocytosis of the targetable glycoconjugates into the cells occurred. Figure 4 shows fluorescence located in round-shaped organelles (lysosomes) situated around the nuclei. Figure 6 reveals a sample polymer located in the lysosomes, consistent with endocytotic subcellular trafficking to these organelles.

Our observation that the copolymers can be internalized into the lysosomal compartments of the cells is similar to findings of a study by Omelyanenko *et al.* (29) who examined the colocalization of FITC-labeled HPMA copolymer conjugate simultaneously with α_2-macroglobulin-RITC marker to visualize the lysosomes in $HepG_2$ cells. The α_2-macroglobulin-RITC, as well as the HPMA-FITC conjugates, accumulated in round-shaped organelles localized in the perinuclear region of cells. It was concluded that colocalization of the lysosomal marker and the FITC-labeled HPMA copolymer conjugate indicates the lysomotropism of HPMA copolymer conjugates.

In addition, the internalization studies as depicted in Fig. 4 shed information regarding the mechanism of internalization of the conjugates. Although all the pictures of cells incubated with the glycoconjugates show significant fluorescence emanating from internalized polymers, the fluorescence from internalized control polymers is comparatively very weak. This difference between the glycoconjugate and the control polymer suggests that the glycoconjugates were endocytosed by a process triggered by the carbohydrate moiety, most likely receptor mediated. It is also possible that the control polymer was endocytosed as well, however, via fluid phase endocytosis, a nonspecific process that does not entail the ligand concentration associated with receptor-mediated endocytosis. Thus, the control polymer was only poorly detected by confocal microscopy during the course of the study.

Although metastatic growth of colon carcinoma cells is associated with increased expression of galactose- and lactose-binding lectins (9–14), we found that the binding of the lactoside-containing HPMA copolymer to all three human colon-adenocarcinoma cells was rather weak. The monovalent galactoside- and the trivalent galactoside-bearing copolymers displayed the strongest fluorescence intensity, which could be attributed to the presence of galectins on the cells' surface. The biorecognition of the monovalent galactoside and the trivalent galactoside was similar, even at a high content of carbohydrate moieties. Because a significant part of this binding (about 50%) was inhibited by lactose, we conclude that the binding was at least partially carbohydrate dependent.

In contrast to what was observed with ASGP-R binding, glycoside cluster effect was not observed toward galectins when trivalent galactoside-containing copolymers were used. A possible explanation could be the fact that galectins possess only a single binding site (monomer membrane protein) (30), as opposed to the ASGP-R, which is composed of three or more subunits that project their CRD extracellularly (2), hence demonstrating a pronounced glycoside cluster effect. Our findings are consistent with the data previously reported

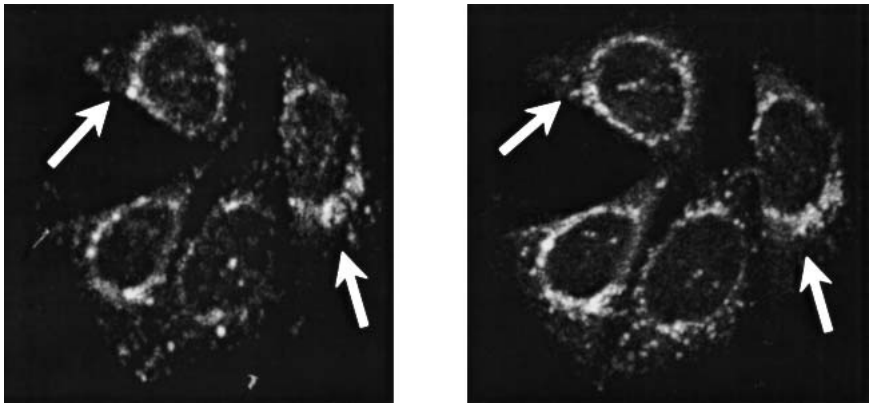

Fig. 6. Confocal fluorescence images of SW-480 cells incubated with the saccharide-containing HPMA copolymer 20% P-TriGal, followed by incubation with the Lysotracker Red DND-99. The left image was taken via an FITC channel. The right image was taken via the Red DND channel. Organelles containing the polymer are morphologically similar to organelles containing the Lysotracker Red. Arrows mark typical two organelles.

by Andre *et al* (24), who showed that cluster design of galactose and lactose residues did not improve the binding affinity toward galectin-1 and galectin-3. To clarify the role of lectins in the biorecognition of the saccharide-containing HPMA copolymers, the expression of galectin-3 binding protein and its specific RNA in the three human colon adenocarcinoma lines (Colo-205, SW-480, and SW-620) is currently being investigated.

Assuming that an increase in overall expression of endogenous lectin is correlated with neoplastic progression of colon-adenocarcinoma (9–14), we hypothesized that the glycosides bearing HPMA copolymers would bound preferentially to metastatic colon adenocarcinoma cells. In contrast to what has been expected, little differences in binding of the glycoconjugates among the cell lines, whether derived from a primary tumor or from a metastatic lesion, were observed. So was the case among cells from a different origin. Perhaps binding studies with more types of colon cancer cell lines could provide more information on the relationship between the β-galactoside-binding capacity of the cells and their differentiation state.

In conclusion, the three human colon adenocarcinoma cell lines examined display β-galactoside-binding capacity. Introduction of galactoside residues into the HPMA copolymer resulted in a significant increase in the binding of the copolymers to the cells. This binding was found to be specific in nature. Clustering design did not generate a remarkable glycoside cluster effect. Mono- and trivalent galactosides showed similar binding intensities for the colon adenocarcinoma cells. Because of their lowest binding capabilities, lactoside-bearing copolymers are poor candidates for targeting drug conjugates to colon-adenocarcinoma cells. The targeting selectivity of galactose-containing copolymers to human colon adenocarcinoma cells requires further investigation.

To examine whether HPMA copolymer-doxorubicin (DOX) conjugates could be targeted to colon cancer cells and serve as a site-specific drug delivery system, we have recently designed three HPMA copolymer-DOX conjugates containing galactose, lactose, and trivalent galactoside. The cytotoxicity of targetable HPMA copolymer-DOX conjugates toward human colon adenocarcinoma is currently being compared with nontargetable HPMA copolymer-DOX conjugates and free DOX. The relationship between the type of the saccharide moieties, the sugar valency, and their anticancer activity will be also addressed.

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