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Effects of natural complex carbohydrate (citrus pectin) on murine melanoma cell properties related to galectin-3 functions

H. INOHARA and A. RAZ*
Cancer Metastasis Program, Michigan Cancer Foundation, Detroit, MI 48201, USA

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Citrus pectin (CP) and pH-modified citrus pectin (MCP) are highly branched and non-branched complex polysaccharides, respectively, rich in galactoside residues, capable of combining with the carbohydrate-binding domain of galectin-3. We reported previously that intravenous injection of B16-F1 murine melanoma cells with CP or MCP into syngeneic mice resulted in a significant increase or decrease of lung colonization, respectively (Platt D, Raz A (1992) *J Natl Cancer Inst* **84**:438–42). Here we studied the effects of these polysaccharides on cell–cell and cell–matrix interactions mediated by carbohydrate-recognition. MCP, but not CP, inhibited B16-F1 melanoma cells adhesion to laminin and asialofetuin-induced homotypic aggregation. Both polysaccharides inhibited anchorage-independent growth of B16-F1 cells in semisolid medium, i.e. agarose. These resuls indicate that carbohydrate-recognition by cell surface galectin-3 may be involved in cell-extracellular matrix interaction and play a role in anchorage-independent growth as well as the *in vivo* embolization of tumour cells.

Keywords: galectin-3; citrus pectin; melanoma.

Abbreviations: CP, natural citrus pectin; MCP, pH-modified CP; EHS, Englebreth–Holm Swarm; CMF-PBS, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, pH 7.2; HRP, horseradish peroxidase; ABTS, 2,2′-azino-di(3-ethylbenzthiazoline sulfonic acid; DMEM, Dulbecco's modified Eagle's minimal essential medium; BSA, bovine serum albumin.

Introduction

Endogenous vertebrate galactoside-binding lectins have been identified and characterized in a diversity of tissues and cells [1]. The lectins are divided into two abundant classes based on their sizes, the molecular masses of which are ~14 kDa and ~30 kDa that have been recently designated as galectin-1 and galectin-3, respectively [2]. Galectin-3 represents a wide range of molecules, i.e. the murine 34 kDa (mL-34) and human 31 kDa (hL-31) tumour-associated galactoside-binding lectins [3, 4], the 35 kDa fibroblast carbohydrate-binding protein (CBP35) [5], the IgE-binding protein (εBP) [6], the 32 kDa macrophage non-integrin laminin-binding protein (Mac-2) [7], and the rat, mouse, and human forms of the 29 kDa galactoside-binding lectin (L-29) [8]. Molecular cloning studies have revealed that the polypeptides are identical. These lectins consist of two structural domains, an amino-terminal domain containing a collagen-like sequence and globular carboxy-terminal domain encompassing the galactoside-binding site [3–8]. Whether all of the above-mentioned galactoside-binding lectins share the same natural ligand(s) is not yet known. Although galectin-3 has been considered to be an S-type lectin that requires reducing conditions for its carbohydrate-binding activity, recent studies have produced evidence to the contrary [9, 10]. Several lines of analysis have demonstrated that the galectins participate in cell–cell and cell–matrix interactions by recognizing and binding complementary glycoconjugates and thereby play a crucial role in various normal and pathological processes [1].

Galectin-3 is highly expressed by activated macrophages and oncogenically transformed and metastatic cells [11, 12]. Elevated expression of the polypeptide is associated with an increased capacity for anchorage-independent growth, homotypic aggregation, and tumour cell lung colonization [13–15], which suggests that galectin-3 promotes tumour cell embolization in the circulation and enhances metastasis. We have previously reported that intravenous injection of citrus pectin (CP) increases lung colonization of the B16-F1 murine melanoma cells, while pH-modified CP (MCP) decreases lung colonization [16]. Although the increased lung colonization by CP is most probably due to its ability to promote homotypic aggregation, the mechanism by which MCP prevents the lung colonization remains less well established.

* To whom correspondence should be addressed.

Laminin, the major non-collagenous component of basement membranes, is an *N*-linked glycoprotein carrying poly-*N*-acetyllactosamine sequences, and is implicated in cell adhesion, migration, growth, differentiation, invasion and metastasis [17, 18]. Galectins which bind with high affinity to oligosaccharides containing poly-*N*-acetyllactosamine sequences [19, 20] also bind to the carbohydrate side chains of laminin in a specific sugar-dependent manner [21, 22].

In order to further study the functional properties of galectin-3, we examined whether CP and MCP would affect galectin-3-related properties of B16-F1 murine melanoma cells. We found that: (a) MCP, but not CP, inhibits cell adhesion to laminin; (b) MCP inhibits asialofetuin-induced homotypic aggregation while CP enhances it; and (c) both CP and MCP inhibit anchorage-independent growth in a semi-solid medium.

Materials and methods

Materials

CP and EHS laminin were purchased from Sigma, St Louis, MO. MCP was prepared from CP by pH modification according to the procedure of Albersheim *et al.* [23]. Asialofetuin was prepared by mild acid hydrolysis of fetuin (Spiro method; Grand Island Biological Co., Grand Island, NY) in 0.05 M H_2SO_4 at 80 °C for 1 h. Recombinant galectin-3 was extracted from bacteria cells by single-step purification through an asialofetuin affinity column as described elsewhere [10]. Recombinant galectin-3 eluted by lactose was extensively dialysed against Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, pH 7.2 (CMF-PBS) before use. Anti-galectin-3 monoclonal antibody was a generous gift from Dr R. Lotan, University of Texas, M. D. Anderson. Horseradish peroxidase (HRP)-conjugated rabbit anti-rat IgG + IgM and 2,2′-azino-di(3-ethylbenzthiazoline sulfonic acid) (ABTS) substrate kit were purchased from Zymed, South San Francisco, CA. B16-F1 murine melanoma cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, non-essential amino acids, 2 mM glutamine, and antibiotics. The cells were maintained at 37 °C in a humidified atmosphere of 7% CO_2 and 93% air.

Cell adhesion to laminin

Tissue culture wells of 96-well plates were precoated overnight at 4 °C with EHS laminin (2 μg per well) in CMF-PBS, and the remaining protein binding sites were blocked for 2 h at room temperature with 1% bovine serum albumin (BSA) in CMF-PBS. Cells were harvested with 0.02% EDTA in CMF-PBS and suspended with serum-free DMEM. 5×10^4 cells were added to each well in DMEM with or without CP or MCP of varying concentrations. After incubation for 2 h at 37 °C, non-adherent cells were washed off with CMF-PBS. Adherent cells were fixed with methanol and photographed. The relative number of adherent cells was determined in accordance with the procedure of Oliver *et al.* [24]. Briefly, the cells were stained with methylene blue followed by the addition of HCl-ethanol to release the dye. The optical density (650 nm) was measured by a plate reader.

Asialofetuin-induced homotypic aggregation

Cells were detached with 0.02% EDTA in CMF-PBS and suspended at 1×10^6 cell per ml in CMF-PBS with or without 20 μg ml^{-1} of asialofetuin and 0.5% CP or 0.5% MCP. Aliquots containing 0.5 ml of cell suspension were placed in siliconized glass tubes and agitated at 80 rpm for 60 min at 37 °C. The aggregation was then terminated by fixing the cells with 1% formaldehyde in CMF-PBS. Samples were used for counting the number of single cells, and the resulting aggregation was calculated according to the following equation: $(1 - N_t/N_c) \times 100$, where N_t and N_c represent the number of single cells in the presence of the tested compounds and that in the control buffer (CMF-PBS), respectively.

Galectin-3 binding to MCP

Ninety-six well plates were coated with CMF-PBS containing 0.5% MCP and 1% BSA and dried overnight. Recombinant galectin-3 serially diluted in CMF-PBS containing 0.5% BSA and 0.05% Tween-20 (solution A) in the presence or absence of 50 mM lactose was added and incubated for 120 min, after which the wells were drained and washed with CMF-PBS containing 0.1% BSA and 0.05% Tween-20 (solution B). Rat anti-galectin-3 in solution A was added and incubated for 60 min, followed by washing with solution B and incubation with HRP-conjugated rabbit anti-rat IgG + IgM in solution A for 30 min. After washing, relative amounts of bound enzyme conjugated in each well were ascertained by addition of ABTS. The extent of hydrolysis was measured at 405 nm.

Colony formation in semi-solid medium

Cells were detached with 0.02% EDTA in CMF-PBS and suspended at 1×10^3 cell per ml in complete DMEM with or without CP or MCP of varying concentrations. The cells were incubated for 30 min at 37 °C and then mixed 1:1 (vol/vol) with a solution of 1% agarose in distilled water-complete DMEM (1:4, vol/vol) preheated at 45 °C. Two ml aliquots of the mixture were placed on top of a precast layer of 1% agarose in 6 cm-diameter dishes. The cells were incubated for 14 days at 37 °C, and the number of formed colonies was determined using an inverted phase microscope after the fixation by the addition of 2.6% glutaraldehyde in CMF-PBS.

Results and discussion

We have previously shown that laminin can serve as a ligand for soluble galectin-3 [22] and that B16-F1 cells

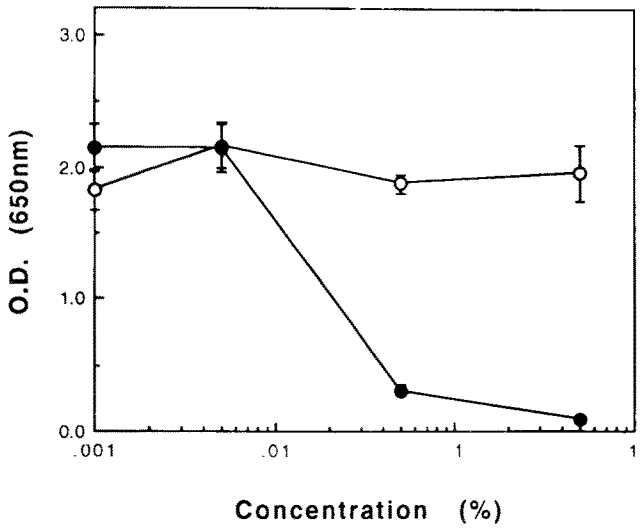

Figure 1. Effects of CP and MCP on B16F1 adhesion to laminin. The cells (5×10^4) were plated on laminin-coated wells (2 μg per well) in the presence of varying concentrations of CP (○) or MCP (●). After 2 h incubation at 37 °C, wells were washed to remove non-adherent cells, stained, and the relative number of adherent cells was determined as described under Materials and methods. All experiments were done in duplicate on two independent occasions. Vertical bars show mean ± SD computed from the *t* distribution of the mean.

express galectin-3 molecules on their cell surface [25]. These results together with the effects of CP and MCP on the lung colonization of i.v. injected B16-F1 cells [16] prompted us initially to examine their effects on B16-F1 cell adhesion to laminin in order to evaluate the possible role of cell surface galectin-3 in such a process. As shown in Figs 1 and 2, MCP significantly inhibited cell adhesion to laminin in a dose-dependent manner, while CP had no apparent effect on either cell binding or spreading on to laminin. Lactose, an inhibitor of galectin-3, did not inhibit cell adhesion to laminin at concentrations as high as 100 mM (data not shown). Competitive binding assay utilizing soluble recombinant galectin-3 failed to block cell adhesion to laminin and the anti-galectin-3 monoclonal antibodies failed in this regard as well (data not shown), suggesting that the inhibitory effect of MCP cannot be attributed solely to its interruption of the interaction between galectin-3 and *N*-acetyllactosaminyl side chains on laminin since cells may utilize the integrins for binding to the protein core of laminin. Furthermore, the anti-galectin-3 monoclonal antibody is not directed against the carbohydrate-binding domain of galectin-3 but rather to its *N*-terminal part thus, the exact mechanism by which MCP blocks adhesion, in contrast to CP and lactose, remains unclear. This inhibitory effect of MCP is not due to cytotoxicity, because MCP (0.5%) did not affect either viability or *in vitro* growth of the cells.

A good correlation has been established between the propensity of tumour cells to undergo homotypic aggregation *in vitro* and their metastatic potential *in vivo* [26]. B16 melanoma cell clumps produce more lung colonies after i.v. injection than do single cells [27]. Moreover, anti-galectin-3 antibody has been shown to inhibit asialofetuin-induced homotypic aggregation [14], suggesting that cell surface galectin-3 brings about the formation of homotypic aggregates following its interaction with the side chains of glycoproteins. As shown in Figs 3 and 4, MCP significantly reduced the formation of homotypic aggregates, while CP enhanced it. Most probably the non-branched MCP mimics

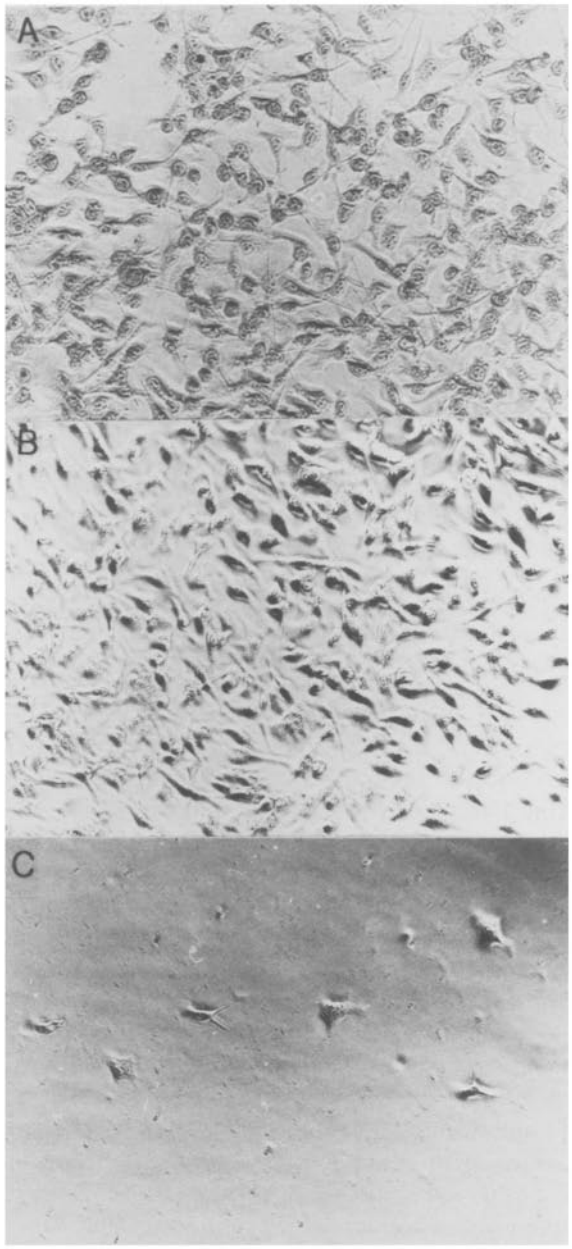

Figure 2. Phase-contrast photographs of B16-F1 cells plated on laminin. The cells were cultured in DMEM alone (A), or in the presence of 0.5% CP (B) or 0.5% MCP (C). Magnification ×200 in all photographs.

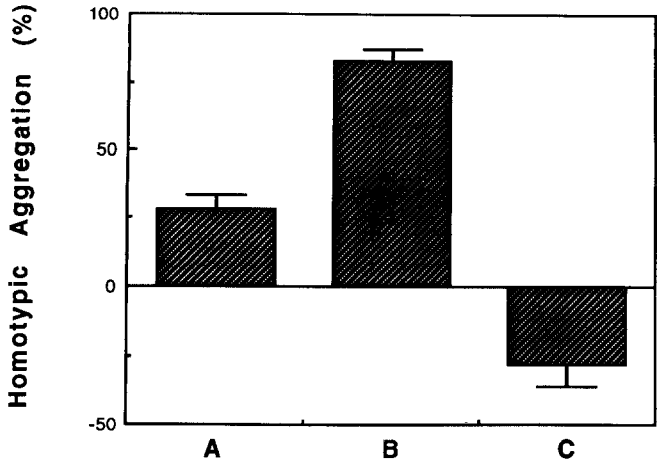

Figure 3. Effects of CP and MCP on asialofetuin-induced homotypic aggregation. The cells were agitated for 60 min at 37 °C, in the presence of 20 μg ml^{-1} asialofetuin alone (A) or with added 0.5% CP (B) or 0.5% MCP (C), and the degree of cell aggregation was determined as described under Materials and methods. All experiments were done in triplicate. Vertical bars show mean ± SD computed from the *t*-distribution of the mean.

the behaviour of the specific sugar inhibitor, i.e. lactose, such that it masks the interaction of the cell surface galectin-3 molecules with galactoside residues of asialofetuin, resulting in a reduced homotypic aggregation. Conversely, it is conceivable to assume that the structural characteristic of a branched carbohydrate polymer allows CP to serve as a cross-linker bridge between adjacent cells, leading to the enhanced formation of homotypic aggregates. Taken together, it may be suggested that MCP could prevent metastasis by disrupting cell–cell and cell–matrix interactions that are crucial for tumour cells to form metastatic lesions.

The aforementioned inhibitory effects of MCP on B16-F1 cell adhesion to laminin and homotypic aggregation may be due to its interaction with galectin-3 on the cell surface, because CP has been previously shown to bind B16-F1 cell surface in a lactose-dependent manner [16]. To address the binding of galectin-3 to MCP, we employed an enzyme-linked immunosorbent assay where we found that recombinant galectin-3 bound immobilized MCP in a dose-dependent manner and the binding was completely blocked by lactose (Fig. 5). These results allow us to attribute the inhibitory effects of MCP on homotypic aggregation to its binding to cell surface galectin-3 molecules. On the other hand, we do not know how MCP, but not CP, impairs B16-F1 cell adhesion to laminin. Since pH modification of CP, which is a branched complex polysaccharide polymer, results in the generation of non-branched carbohydrate chains of the same sugar composition, it is likely that MCP binds more avidly to the cell surface galectin-3 molecules than does CP. Taken together with the fact that anti-integrin antibodies inhibit murine B16 melanoma cell

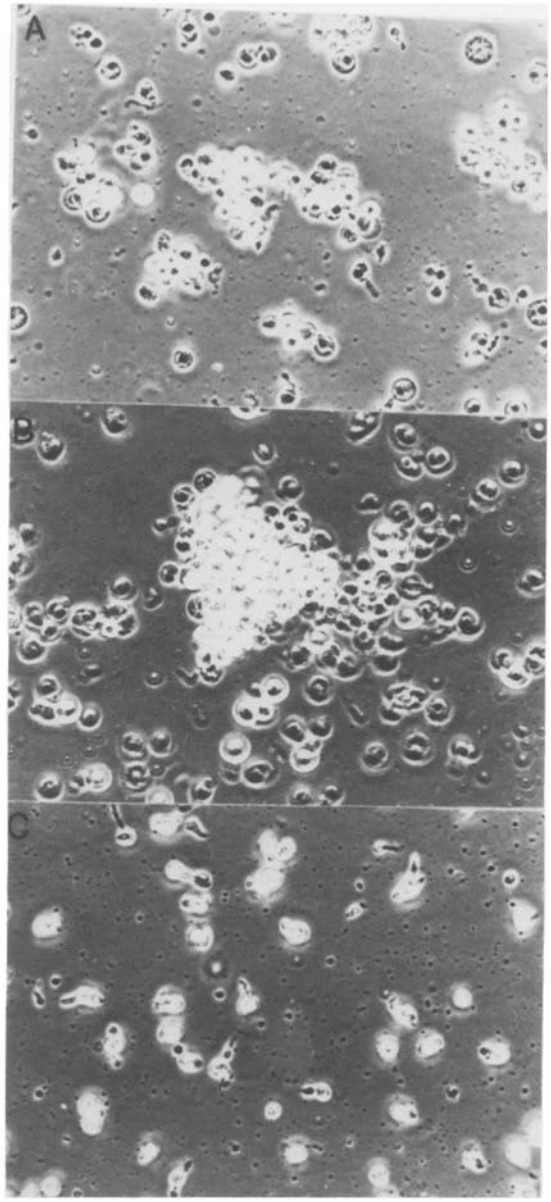

Figure 4. Phase-contrast photographs of homotypic aggregation of B16-F1 cells. The cells were allowed to aggregate in the presence of 20 μg ml^{-1} asialofetuin alone (A) or with added 0.5% CP (B) or 0.5% MCP (C). Magnification ×200 in all photographs.

attachment to laminin substrates [28], we presume that MCP sterically inhibits laminin recognition by the integrin class of laminin receptors, or that the interaction of cell surface galectin-3 with poly-*N*-acetyllactosamine sequences on laminin may act in concert with integrins for cell adhesion to laminin. The possibility that the interaction of MCP with galectin-1 having the same sugar specificity as galectin-3 might affect its processes to impair B16-F1 cell adhesion to laminin and homotypic aggregation can be most probably ruled out since galectin-1 is a secreted protein [29].

The ability of cells to grow in semi-solid medium, i.e.

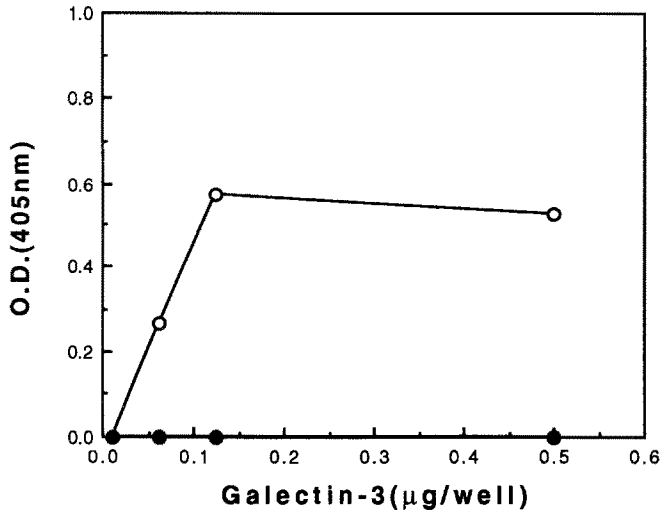

Figure 5. Binding of galectin-3 to MCP coated wells. Binding of recombinant galectin-3 in a dose-dependent manner to MCP coated wells in the absence (○) or presence (●) of 50 mM lactose was assayed as described under Materials and methods. Each determination represents the mean of duplicate experiments.

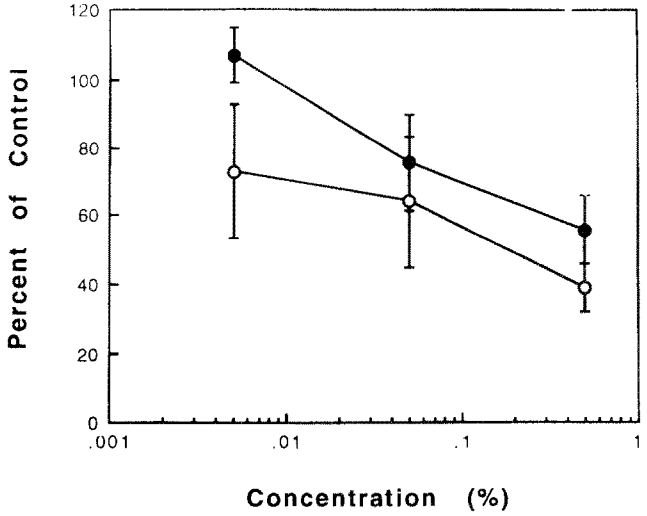

Figure 6. Effects of CP and MCP on the ability of B16-F1 cells to form colonies in 0.5% agarose. The cells were incubated for 30 min at 37 °C in the presence of CP (○) or MCP (●) of varying concentrations and then mixed 1:1 with 1% agarose. 1×10^3 cells were plated and incubated at 37 °C for 14 days. The number of colonies was determined in pentaplicate on two independent experiments, and shown as a percentage compared to that in the absence of the compounds with a SD bar.

'anchorage independence' is used as a criterion for cell transformation, because this property is usually exhibited only by transformed and tumorigenic cells [30–32]. Previously it has been suggested that the ability of tumour cells to interact with protein-bound carbohydrate residues via cell surface galectin-3 is related to their ability to interact with the galactose residues of agarose (a polymer of D-galactose and L-anhydrogalactose) and to the efficiency of colony formation in this semi-solid medium [12]. It has been also shown that anti-galectin-3 monoclonal antibodies inhibit growth of tumour cells in agarose and that there is an inverse relationship between the expression of galectin-3 and the suppression of the transformed phenotype [13]. Transfection of normal mouse fibroblast with the mouse galectin-3 cDNA results in the acquisition of anchorage-independent growth properties [33]. To further verify the possibility that cell surface galectin-3 play a key role for cells to grow in semi-solid medium, we examined the effects of CP and MCP on anchorage-independent growth of B16-F1 melanoma cells. As shown in Fig. 6, CP and MCP inhibited the growth of B16-F1 cell colonies in the semi-solid matrix in a dose-dependent manner. Similarly, lactose inhibited anchorage-independent growth in a dose-dependent manner as well (data not shown). The dose-dependent inhibitory effect of CP and MCP was not restricted to B16-F1 melanoma cells. The growth in soft agar of UV-2237-Ip-3 murine fibrosarcoma cells, HT1080 human fibrosarcoma cells, and A375CI.49 human melanoma cells was also equally inhibited (data not shown). It is possible that the soluble CP and MCP compete with the galactose residues of agarose for galectin-3 binding, leading to apparent growth inhibition by depriving the cells of the minimal support of the matrix required for cell proliferation.

It also may be argued that CP and MCP as well as the anti-galectin-3 antibodies possibly behave like an antagonist of an as-yet unrecognized glycoconjugate growth factor which interacts with galectin-3, or they sterically hinder the access of known growth factors to the membrane receptors. However, the fact [16] that *in vitro* anchorage-dependent growth and tumorigenicity of B16-F1 cells in syngenic mice were not impaired by MCP (0.5%) plausibly enables us to rule out the aforementioned possibilities. Since the ability of cells to grow in semi-solid medium is used as a criterion for cell transformation [32], the acquisition of cell surface galectin-3 might be an early step of the post-transformed cascade.

From the results presented here we may draw the following conclusions: (a) the cell surface galectin-3 molecules probably do not function as a laminin-binding receptor, although soluble galectin-3 molecules do bind to laminin; (b) they do play a key role in homotypic aggregation and anchorage-independent growth of tumour cells.

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