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Tetra- and hexavalent mannosides inhibit the pro-apoptotic, antiproliferative and cell surface clustering effects of concanavalin-A: Impact on MT1-MMP functions in marrow-derived mesenchymal stromal cells

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Mesenchymal stromal cells (MSC) mobilization and recruitment by experimental vascularizing tumors involves membrane type 1-matrix metalloproteinase (MT1-MMP) functions. Given that the mannose-specific lectin Concanavalin-A (ConA) induces MT1-MMP expression and mimics biological lectins/carbohydrate interactions, we synthesized and tested the potential of 11 mannoside clusters to block ConA activities on MSC. We found that tetra- and hexavalent mannosides reversed ConA-mediated changes in MSC morphology and antagonized ConA-induced caspase-3 activity and proMMP-2 activation. Tetra- and hexavalent mannosides also inhibited ConA- but not the cytoskeleton disrupting agent Cytochalasin-D-induced MT1-MMP cell surface proteolytic processing mechanisms, and effects on cell cycle phase progression. The antiproliferative and pro-apoptotic impact of ConA on the MT1-MMP/glucose-6-phosphate transporter signaling axis was also reversed by these mannosides. In conclusion, we designed and identified glycocluster constructions that efficiently interfered with carbohydrate-binding proteins (lectins) interaction with oligosaccharide moieties of glycoproteins at the cell surface of MSC. These glycoclusters may serve in carbohydrate-based anticancer strategies through their ability to specifically target MT1-MMP pleiotropic functions in cell survival, proliferation, and extracellular matrix degradation.

Keywords: cancer/concanavalin-A/mannosides/mesenchymal stromal cells/MT1-MMP

Introduction

Bone marrow-derived mesenchymal stromal cells (MSC) are a population of pluripotent adherent cells residing within the bone marrow microenvironment and have an ability to differentiate into many mesenchymal phenotypes (Prockop 1997; Horwitz et al. 2005). Interestingly, the recruitment of MSC by experimental vascularizing tumors has resulted in the incorporation of MSC in the tumor architecture (Studeny et al. 2004) and implies that these cells must respond to tumor-derived growth factor cues (Annabi, Naud et al. 2004; Birnbaum et al. 2007). Membrane type 1-matrix metalloproteinase (MT1-MMP), an important protein related to tumor growth and angiogenesis, is expressed on malignant tumor cells and in activated endothelial cells (Sato et al. 2005). Among its known activities, MT1-MMP is involved in cell migration, extracellular matrix (ECM) degradation, and endothelial cell tubulogenesis (Genis et al. 2006). Detailed studies by RNA interference recently revealed that MT1-MMP similarly controls human MSC mobilization and homing processes which also require invasion through ECM barriers (Ries et al. 2007). Among the signaling molecules thought to regulate MT1-MMP biological functions, Egr-1 and Wnt regulate MT1-MMP gene expression in chemotaxis and in the invasion capacity of MSC out of the bone marrow (Annabi, Lee, et al. 2003; Neth et al. 2006; Currie et al. 2007). On the other hand, increased expression of MT1-MMP correlated with decreased cell survival in part attributable to a decrease in expression of an endoplasmic reticulum-embedded glucose-6-phosphate transporter (G6PT) (Belkaid et al. 2007; Currie et al. 2007). While the activities of MT1-MMP in tumorigenesis are well documented, its potential role in cell–cell interaction and in MSC engraftment processes remains poorly understood, and new strategies to target MT1-MMP-mediated processes needed to be investigated.

We have previously shown that MSC chemotaxis and cell survival are regulated, in part, by MT1-MMP (Currie et al. 2007). Because of MT1-MMP's ability to promote directed cell migration across reconstituted basement membranes both in metastasis and in tumor angiogenesis processes, newly developed strategies, including specific immunoliposomal anticancer MT1-MMP targeting (Atobe et al. 2007) and interference RNA technology (Arroyo et al. 2007), are currently envisioned. The design, use, and evaluation of these approaches have however been more complex than expected. Although tumor endothelial cells are increasingly accepted as a valid target for cancer therapy, since angiogenesis is a critical event for the maintenance, proliferation, and metastasis of tumors (Brandwijk et al. 2007), none of the above strategies has looked at the impact of MT1-MMP targeting on circulating MSC in response to tumor growth factors remains unknown.

Given the known tendency of transformed cells to express selective carbohydrate motifs in the form of glycoproteins or glycolipids, the design of carbohydrate-based anticancer vaccines

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has recently found therapeutic applications and was developed around the multivalent or cluster effect concept designed to interfere with carbohydrate molecular recognition (Danishefsky and Allen 2000; Roy 2004; Verez-Bencomo et al. 2004). Interactions between carbohydrate-binding proteins (lectins) and the oligosaccharide moieties of glycoprotein at the cell surfaces are, in fact, involved in extensive cellular recognition processes including development, differentiation, morphogenesis, and cell migration. The lectin from *Canavalia ensiformis* (Concanavalin-A, ConA), one of the most abundant lectins known (Lin and Levitan 1991), thus enables one to mimic biological lectin/carbohydrate interactions that regulate ECM protein recognition and, as such, is routinely used to trigger MT1-MMP-mediated activation of latent proMMP-2 (Yu et al. 1997; Zucker et al. 2002; Lafleur et al. 2006). ConA was also found to increase the sub-G1 cell cycle phase as well as cell death in MSC, indicative of a potential role in cell surface clustering that affects cell survival (Currie et al. 2007). Furthermore, MT1-MMP gene silencing significantly abrogated MSC chemotaxis in response to the tumorigenic growth factor sphingosine 1-phosphate in MSC, suggesting a crucial role for that MMP in signaling cell mobilization (Annabi, Thibeault, et al. 2003; Currie et al. 2007).

In this study, generation of several oligomannoside clusters was performed in order to optimize mannan's ability to inhibit ConA actions using synthesis strategies detailed previously (Roy and Touaibia 2007; Touaibia, Shiao, et al. 2007; Touaibia, Wellens, et al. 2007). Structure-function studies were initiated to evaluate their biological abilities to inhibit ConA-induced MT1-MMP-mediated proMMP-2 activation, cell death, and antiproliferative property in MSC. As model substances for effectively functioning as biological response modifiers, the cell-binding capacity of lectins, such as ConA, enables the dissection of cellular processes involved in morphogenesis, ECM degradation, and cell death. We show that specific tetra- and hexavalent mannoside clusters very effectively inhibit a spectrum of MT1-MMP-mediated cell responses that could be potentially transposed to target tumor-promoting processes.

Results

Chemical structures of the mannosides tested

Pentaerythritol and bis-pentaerythritol scaffolds were previously used toward the preparation of a series of tri- to hexaclusters bearing α-D-mannopyranoside residues assembled using single steps "Sonogashira coupling" and "click chemistry." The carbohydrate precursors were built with either para-iodophenyl, propargyl, or 2-azidoethyl aglycones while the pentaerythritol moieties were built with terminal azide, propargyl, or para-iodophenyl groups (Roy and Touaibia 2007; Touaibia, Shiao, et al. 2007; Touaibia, Wellens, et al. 2007). These clusters were initially chosen for their ability to cross-link Concanavalin-A (ConA) and have already shown to be 1000 times more potent than mannose for their capacity to inhibit the binding of fimbriated *E. coli* expressing mannoside binding pili lectin to erythrocytes in vitro (Touaibia, Wellens, et al. 2007). Whether any of these clusters could further compete against the cellular and molecular effects of ConA was assessed below. The chemical structure of the different mannosides is depicted in Figure 1.

Mannan and mannosides DM58, DM54, and DM75 prevent Concanavalin-A-induced changes in cell morphology

Disruption of the cytoskeleton architecture is among the first cellular events known to be affected upon treatment with ConA Lin and Levitan 1991; Allenberg et al. 1994; Zanetta et al. 1994; Belkaid et al. 2007). MSC were serum-starved and treated with ConA in the presence or absence of the different mannosides shown in Figure 1 as described in the Materials and methods section. Phase contrast pictures were taken and cell aggregation was noticed in ConA-treated MSC (Figure 2A, control). Mannan, DM58, DM54, and DM75 were the only mannosides to significantly prevent the ConA-induced aggregating effect, suggesting that these four molecules may efficiently inhibit cell surface ConA-binding biological effects. The potency to inhibit ConA-induced hemagglutination of erythrocytes was performed with serial 2-fold dilutions of the synthesized molecules (Dam et al. 1998). The lowest concentration of the molecules required to completely inhibit ConA-induced red blood cells agglutination was determined visually and clearly demonstrates the high agglutination inhibitory effect of DM58 in comparison to all other molecules tested, which was ∼8 times more potent than DM71 and DM75, and ∼16 times more potent than DM54 (see squared wells, Figure 2B). In order to further assess the direct lectin binding ability of Mannan, DM58, DM54, and DM75 molecules (DM71 was discarded because of its inability to reverse ConA-induced cell aggregation) ConA was treated with each of the clusters at 1 mg/mL in microtiter plates using the polysaccharide yeast mannan as positive control. Rapidly and within 5 min, insoluble cross-linked complexes were formed as judged by the cloudiness within the wells (not shown). The optical density (O.D.) was measured at 490 nm as previously described (Touaibia, Wellens, et al. 2007) and demonstrated a significant ConA binding activity of the synthetic glycoclusters (Figure 2C). The results clearly illustrate the rapid preference of tetraaryl mannoside DM58, obtained via the Sonagashira coupling, to form a cross-linked lattice toward ConA, over the alternate functional isomer DM54 or hexamer DM75. The latter two possessed however comparable lectin binding activities to the reference yeast mannan.

Mannan and mannosides DM58, DM54, and DM75 inhibit Concanavalin-A-induced proMMP-2 activation

Cytoskeleton disrupting agents ConA and Cytochalasin-D (CytoD) trigger latent proMMP-2 activation into its active MMP-2 form (Gingras et al. 2000). Serum-starved MSC were treated with either ConA or CytoD in the presence or absence of the different mannosides as described in the Materials and methods section. Conditioned media were then harvested and assessed for latent proMMP-2 and active MMP-2 content by gelatin zymography. While none of the mannosides was able to antagonize CytoD-induced proMMP-2 activation (Figure 3A), the mannosides DM58, DM54 and DM75, in contrast, very efficiently (better even than mannan) inhibited ConA-induced proMMP-2 activation (Figure 3B). A dose response analysis of the inhibitory effect of the best mannosides against ConA-induced proMMP-2 activation was assessed by gelatin zymography. Each of the mannan, DM58, DM54, and DM75 mannosides dose-dependently inhibited proMMP-2 activation by ConA, resulting in the progressive disappearance of the active MMP-2 form concomitant to the reappearance of the latent proMMP-2 form (Figure 4A).

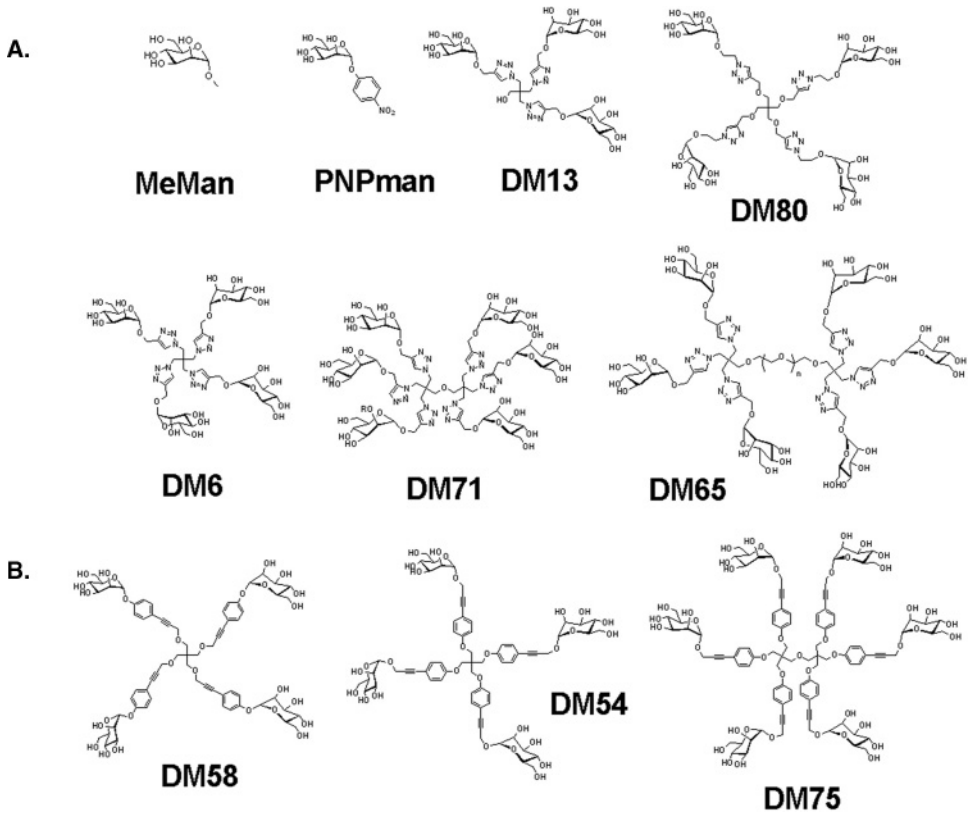

Fig. 1. Chemical structures of the mannosides tested against Concanavalin-A. (**A**) Methylmannose (MeMan), p-nitrophenylmannoside (PNPMan), and mannoside clusters synthesized by Cu(I)-catalyzed [1,3]-dipolar cycloadditions using pentaerythritol scaffolds bearing either alkyne or azide functionalities are represented. (**B**) Mannoside clusters synthesized by palladium-catalyzed Sonogashira reactions using pentaerythritol scaffolds bearing either alkyne or p-iodophenyl functionalities are depicted.

The anti-ConA effects of the mannosides tested enabled us to calculate the IC_{50} values for each of them as follows: Mannan 8.2 $\pm$ 0.9 μg/mL, DM58 1.3 $\pm$ 0.4 μg/mL, DM54 2.6 $\pm$ 0.2 μg/mL, and DM75 5.1 $\pm$ 1.1 μg/mL, establishing DM58 $\sim$8 times more potent than mannan for inhibiting ConA-induced proMMP-2 activation and confirming the turbidimetric data obtained in Figure 2B. These results suggest that efficient inhibition of proMMP-2 activation by mannosides may account for decreased ECM degradation capacity and subsequent mobilization of MSC.

Mannan and mannosides DM58, DM54, and DM75 inhibit Concanavalin-A-induced caspase-3 activity

ProMMP-2 activation has recently been correlated to cell death (Preaux et al. 2002; Hinoue et al. 2005). Since ConA-induced proMMP-2 activation is thought to be mediated through a MT1-MMP-dependent process in MSC (Currie et al. 2007), we further investigated the ability of the above-identified best four mannosides to antagonize ConA- and CytoD-induced cell death. Serum-starved MSC were thus treated as described above in the presence or absence of mannan, DM58, DM54, and DM75 mannosides. Cells were then harvested as described in the Materials and methods section in order to assay caspase-3 activity. We found that ConA and CytoD significantly induced cell death and caspase-3 activity (Figure 5) as previously documented (Annabi, Thibeault, et al. 2003; Belkaid et al. 2007; Currie et al. 2007). Intriguingly, while mannan and the mannosides DM58, DM54, and DM75 completely prevented caspase-3 activation in ConA-treated MSC (Figure 5, black bars), only DM58 was efficient at doing so in CytoD-treated MSC (Figure 5, gray bars). Because CytoD directly inhibits intracellular actin polymerization (Lambert et al. 2001; Sanka et al. 2007; Yourek et al. 2007), this observation suggests that, except for DM58, the mannosides tested specifically antagonize ConA cell surface clustering in MSC. Collectively, the inhibition of ConA-induced cell morphology, proMMP-2 activation, and cell death by the mannosides prompted us to further explore the involvement of mannosides against MT1-MMP functions.

Mannan and mannosides DM58, DM54, and DM75 inhibit Concanavalin-A-induced cell surface MT1-MMP proteolytic processing and affect the MT1-MMP/G6PT signaling axis

Among the early events that take place upon cytoskeleton disorganization is the activation of proMMP-2 by MT1-MMP. Given evidence that endoplasmic reticulum (ER) dysfunction is often linked to cytoskeleton perturbations and to cell death signaling, a molecular signaling axis between MT1-MMP and the ER-embedded G6PT was highlighted in MSC (Currie et al. 2007). We thus sought to investigate whether the different mannosides generated were able to regulate ConA-induced MT1-MMP cell surface processing, and if the MT1-MMP/G6PT signaling axis was affected. We found that MSC treated with either ConA or CytoD exhibited an increase in the active 55 kDa form of MT1-MMP and in the appearance of its 43 kDa inactive proteolytic

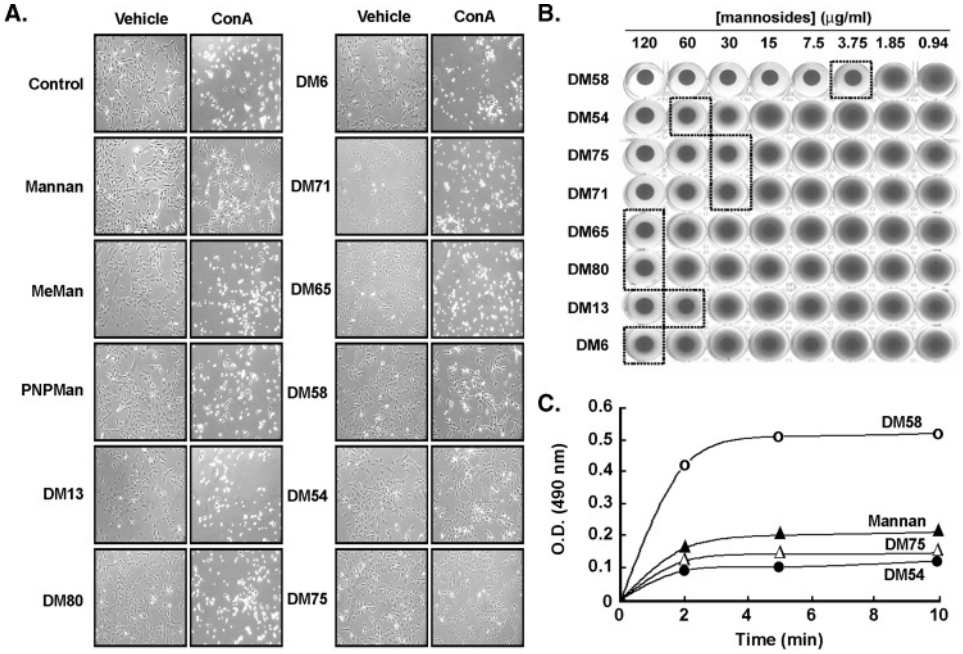

Fig. 2. Mannan and mannosides DM58, DM54, and DM75 prevent Concanavalin-A-induced changes in cell morphology through direct cross-linking properties. (**A**) Subconfluent MSC were serum-starved and treated with 10 μg/mL ConA or vehicle (water) in the presence or absence of 10 μg/mL of the different mannosides described in Figure 1 for 18 h at 37°C. Phase contrast pictures were taken, and cell aggregation was reflected by rounding in the cell morphology. (**B**) Inhibition of ConA-mediated hemagglutination of erythrocytes potency was tested as described in the Materials and methods section for all the mannosides synthesized. Squared wells show the minimum concentration required for inhibition from a representative experiment ($n = 3$). (**C**) The classical time course of the microtiter plate turbidimetric analysis (microprecipitation) of ConA was performed as described previously (Touaibia, Wellens, et al. 2007) in the presence of mannosides DM58 (open circle), DM54 (closed circle), DM75 (open triangle), and yeast mannan (closed triangle) as positive control. Measurements were performed in PBS at 1 mg/mL ConA using an ELISA plate reader at 25°C. Optical density (O.D.) was read at 490 nm, and data are the average of triplicate values.

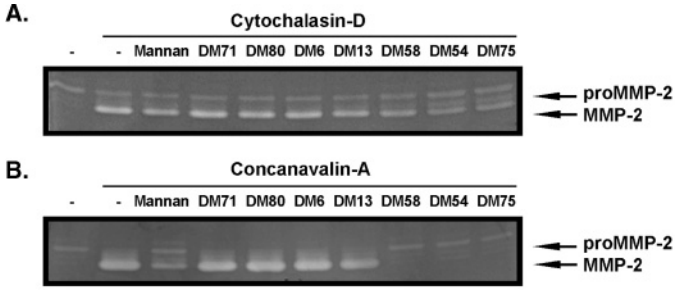

Fig. 3. Mannan and mannosides DM58, DM54, and DM75 inhibit Concanavalin-A- but not Cytochalasin-D-induced proMMP-2 activation. Subconfluent MSC were serum-starved and treated with either (**A**) 1 μM CytochalasinD (CytoD) or (**B**) 10 μg/mL ConA in the presence or absence of 10 μg/mL of the different mannosides described in Figure 1 for 18 h at 37°C. Conditioned media were then harvested and gelatin zymography performed as described in the Materials and methods section in order to monitor the extent of latent proMMP-2 activation into MMP-2.

fragment (Figure 6, upper panels). When MSC were co-treated with mannan, DM58, DM54, or DM75, ConA-induced MT1-MMP proteolytic processing was inhibited while it remained unaffected in CytoD-treated cells (Figure 6, upper panels). These observations are in agreement with those obtained with proMMP-2 activation using gelatin zymography (Figure 3). When the MT1-MMP/G6PT signaling axis was investigated, we found that ConA, but not CytoD, inhibited G6PT expression (Figure 6, lower panels). While mannan, DM58, DM54, and DM75 reversed the G6PT inhibition, they had no effect on G6PT expression in CytoD-treated cells (Figure 6, lower panels). Expression of the extracellular signal-regulated protein kinase (ERK) was found constant throughout the experimental conditions and used as an internal control. Collectively, the mannosides tested efficiently re-established the MT1-MMP/G6PT intracellular signaling axis that was disrupted upon ConA treatment.

The pro-survival effects of Mannoside DM58 prevent the induction of sub-G1 by ConA and CytoD and the decrease in G0/G1 cell cycle phases by Concanavalin-A

The pro-survival impact of mannoside DM58 was further investigated in terms of cell cycle progression. Because proMMP-2 activation is thought to interfere with cell survival and proliferation (Preaux et al. 2002; Hinoue et al. 2005), we also sought to investigate the effect of ConA and CytoD on BMSC cell cycle progression. Cells were treated with ConA or CytoD in the presence or not of DM58, staining was performed with propidium iodine (PI), and flow cytometry was used to assess the proportion of cells found in the G0/G1, S, G2/M, and sub-G1 cell cycle phases (Figure 7A). While DM58 itself had no effects on cell cycle phases, ConA triggered a significant decrease in G0/G1 and CytoD treatment resulted in a combined decrease in G0/G1 and increase in G2/M cell cycle phases (Figure 7A), and that was concomitantly observed with an increase in sub-G1 (Figure 1D). Both ConA and CytoD triggered the appearance of a sub-G1 cell population in the absence of mannosides (Figure 7B, white bars) indicative of cell death and consistent with their pro-apoptotic effects (Figure 5). In accordance with its

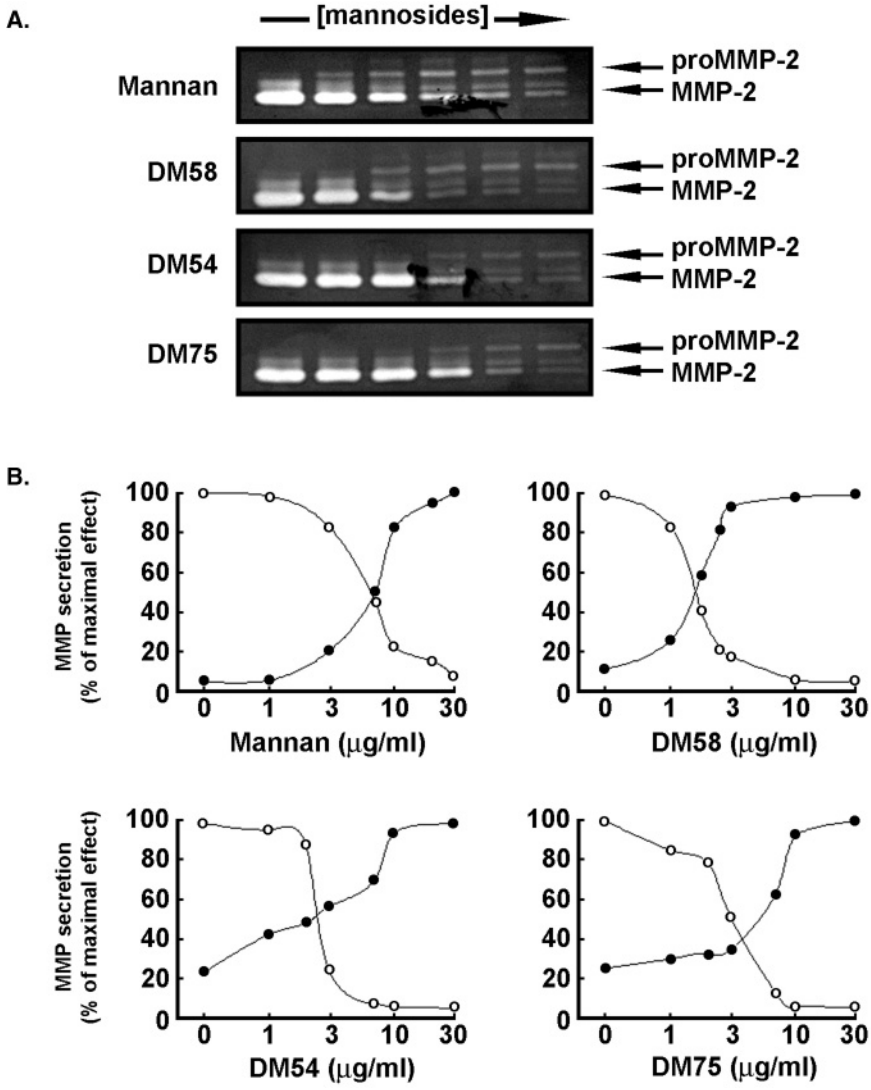

Fig. 4. Differential potencies of mannan and mannosides DM58, DM54, DM75 for inhibiting Concanavalin-A-induced proMMP-2 activation. Subconfluent MSC were serum-starved and treated with increasing concentrations (0–30 μg/mL) of mannan, DM58, DM54 and DM75 in the presence of 10 μg/mL ConA for 18 h at 37°C. (**A**) Conditioned media were then harvested and gelatin zymography performed as described in the Materials and methods section in order to monitor the extent of latent proMMP-2 activation into MMP-2. (**B**) Scanning densitometry of a representative experiment (out of three) was used to quantify the extent of gelatin hydrolysis by the latent proMMP-2 (closed circles) and by the active MMP-2 form (open circles).

pro-survival property, the only mannoside DM58 that was able to protect from ConA- or CytoD-induction of caspase-3 was able to reduce the sub-G1 cell population (Figure 7B, black bars) that appeared upon ConA or CytoD treatment confirming its more complex mechanistic actions.

Discussion

Cell surface carbohydrate structures acting as ligands for tissue specific mammalian lectins have long been recognized in the regulation of cell–cell interactions, particularly in processes such as lymphocyte homing to specific tissues (Stoolman and Rosen 1983), homing of hematopoietic stem cells (Aizawa and Tavassoli 1988), and tumor cell metastasis (Kannagi et al. 2004). As such, lectins on microvascular endothelial cells have also been shown to contribute to retention and secondary tumor formation of blood-borne tumor cells (Cornil et al. 1990). Recent evidence from our laboratory indicates that circulating vascular progenitors derived from murine MSC are recruited by vascularizing tumors (Annabi, Naud, et al. 2004) and that a hypoxic environment, such as that encountered within tumor masses, regulates MSC angiogenic properties (Annabi, Lee, et al. 2003). In vivo, subcutaneous co-injection of MSC with U-87 glioma cells into nude mice resulted in the formation of highly vascularized tumors, where differentiated MSC localized at the lumen of vascular structures (Annabi, Naud, et al. 2004). Although the specific implications of MT1-MMP in cell–ECM and cell–cell interactions that would promote engraftment and potential tumor development remain to be addressed in vivo, these data suggest that MSC can be recruited at the sites of active tumor neovascularization and prompted us to investigate new routes

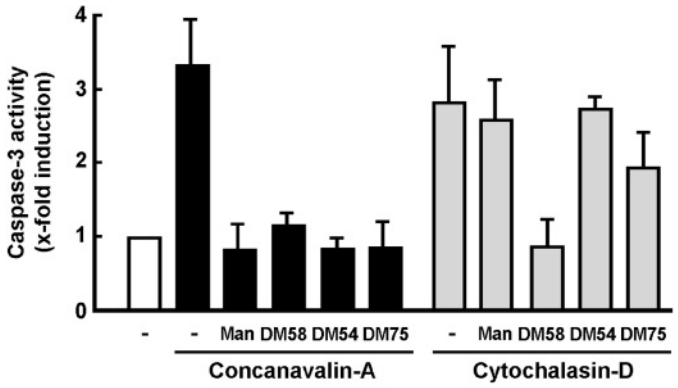

Fig. 5. Mannan and mannosides DM58, DM54 and DM75 inhibit Concanavalin-A-induced caspase-3 activity. Subconfluent BMSC were serum-starved and treated with 10 μg/mL of either mannan, DM58, DM54, or DM75 in the presence of 10 μg/mL ConA (black bars) or 1 μM CytoD (gray bars) for 18 h at 37°C. Caspase-3 activity is shown from a representative experiment and was measured as described in the Materials and methods section in triplicate.

to the use of MT1-MMP functions as a therapeutic target by carbohydrate scaffolds.

The present study revolves around the well-documented ability of the lectin ConA to efficiently trigger MT1-MMP-mediated proMMP-2 activation and cell death signaling. As such, silencing of the MT1-MMP gene prevented ConA-mediated proMMP-2 activation and cell death in MSC (Belkaid et al. 2007; Currie et al. 2007) demonstrating the crucial role that MT1-MMP plays in transducing cell signaling upon cytoskeleton remodeling. MT1-MMP can further function as a signaling molecule as it cooperates with tumor-derived growth factors to induce actin stress fibers and to trigger MSC migration (Meriane et al. 2006). Among the signal transduction events reported, tyrosine phosphorylation is considered a major event upstream of MT1-MMP induction upon ConA cell surface clustering in platelets (Torti et al. 1995), monocytes (Matsuo et al. 1996), neutrophils (Ohta et al. 1992), and breast carcinoma cells (Yu et al. 1997), while activation of the ERK cascade by MT1-MMP represents an important downstream event (Gingras et al. 2001). Additionally, MT1-MMP-mediated intracellular ERK phosphorylation was found crucial in regulating cell–ECM interaction through CD44, thereby regulating cell homing and engraftment (Annabi, Thibeault, et al. 2004; Krause et al. 2006). The cytoskeleton also plays important roles in cell morphology, growth, and signaling during MSC differentiation (Yourek et al. 2007). Changes in the cytoskeleton allow the cell to migrate, divide, and maintain its shape in response to external mechanical stimuli (Hayakawa et al. 2001). Maintenance of cytoarchitecture is, on the other hand, also required for cell survival, since its perturbation by CytoD- or ConA-mediated MT1-MMP mechanisms diminished cell survival and was correlated to proMMP-2 activation (Preaux et al. 2002; Hinoue et al. 2005; this study).

Considerable literature on the binding capacity of aromatic or clustered glycosides to ConA have enabled to dissect signaling cascades relevant to numerous cellular processes such as in cell aggregation and adhesion (Dam et al. 2000; Jain et al. 2000; Dam and Brewer 2003; Li et al. 2004). Among others, these include activation of immune cells, modulation of cytokine secretion, and induction of cell apoptosis (Timoshenko et al. 2000; Andre et al. 2001). In fact, the role of cell surface carbohydrates in ConA-mediated cytoskeletal changes and induction of apoptosis was documented in fibroblasts (Kulkarni and McCulloch 1995). In the present study, the use of cytoskeleton disrupting agents ConA and CytoD effectively triggered MT1-MMP-mediated cell death. Interestingly, while DM58, DM54, and DM75 mannosides antagonized ConA induction of caspase-3 activity, only DM58 was able to reverse CytoD-induced caspase-3, suggesting a direct and more complex prosurvival effect of that mannoside which remains to be investigated. Accordingly, cytoskeleton disorganization is considered an early step in the activation process of proMMP-2 by MT1-MMP, but is also associated with ER dysfunction and subsequent cell death. Given evidence that the ER-embedded G6PT regulates cell survival and that impaired chemotaxis was recently observed in bone marrow cells isolated from a G6PT$^{-/-}$ mouse model (Kim et al. 2006), a MT1-MMP/G6PT signaling axis was recently shown to

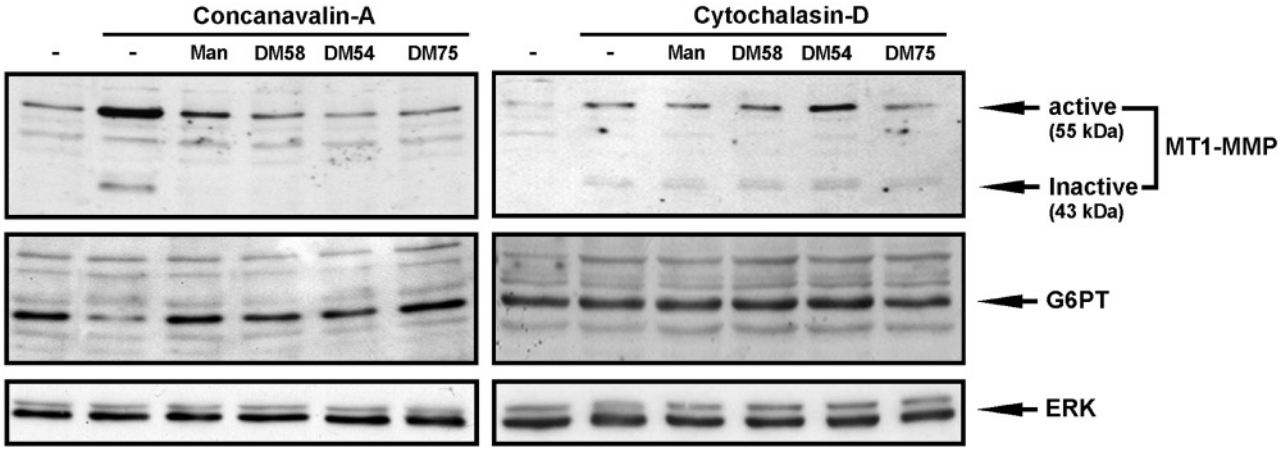

Fig. 6. Mannan and mannosides DM58, DM54 and DM75 inhibit Concanavalin-A-induced cell surface MT1-MMP proteolytic processing and affect the MT1-MMP/G6PT signaling axis. Subconfluent MSC were serum-starved and treated with 10 μg/mL of either mannan, DM58, DM54, or DM75 in the presence of 10 μg/mL ConA (left panel) or 1 μM CytoD (right panel) for 18 h at 37°C. Cell lysates were isolated from each condition and Western blotting followed by immunodetection were performed with anti-MT1-MMP, anti-G6PT, and anti-ERK antibodies as described in the Materials and methods section.

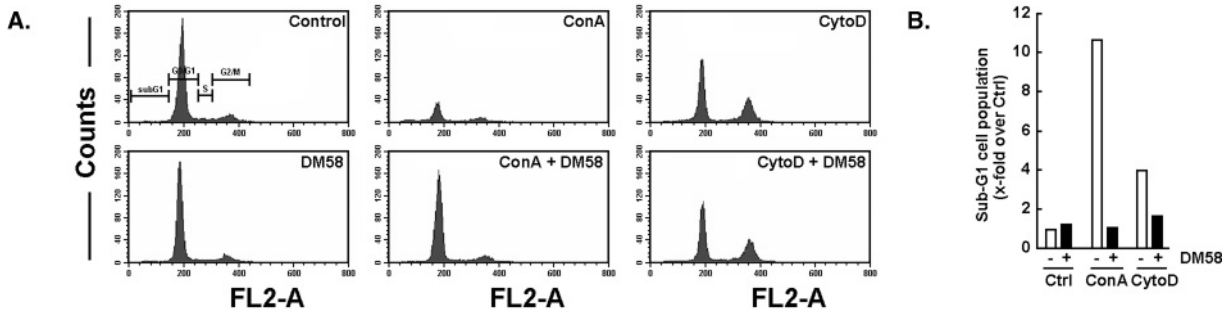

Fig. 7. The pro-survival effects of Mannoside DM58 prevent the induction of sub-G1 by ConA and CytoD, and prevent the decrease in G0/G1 cell cycle phases by Concanavalin-A. BMSC were serum-starved for 24 h and cell cycle phases synchronozed. (**A**) Cells were treated with 10 mg/mL ConA, or 1 μM CytoD, in the presence or not of 10 μg/mL DM58, and cell cycle was analyzed by flow cytometry as described in the Materials and methods section. A representative experiment out of three shows cells present in each division phase. For the sake of clarity, gating for the sub-G1, G0/G1, S, and G2/M cell cycle phases are shown in the Control (Ctrl) picture. (**B**) A representative sub-G1 cell population is depicted upon ConA or CytoD treatments in the absence (white bars) or presence (black bars) of DM58.

link MSC survival, ECM degradation, and mobilization (Currie et al. 2007).

Important progress has been made in the development of carbohydrate scaffolds for drug discovery. Carbohydrates have been proven as valuable scaffolds to display pharmocophores, and the resulting molecules have demonstrated useful biological activity toward various targets including the somatostatin receptors, integrins, HIV-1 protease, MMP, multidrug resistance-associated protein, and as RNA binders and have shown antibacterial and herbicidal activity (Becker et al. 2006). In our study, we used carbohydrates as scaffolds to display chemical functionalities that have the potential to interact with carbohydrate-recognizing receptors. We found that the spatial arrangement of the clusters having the triazole or the phenyl rings appeared to be crucial for their affinity against ConA activities, thus illustrating the influence of multivalency on this scaffold. The introduction of four or six mannopyranoside moieties using extended precursors and 1,3-cycloaddition had a minor effect on the relative affinity, since compounds DM13, DM6, DM80, and DM65 having respectively three, four, and six mannoside residues, were almost equipotent (Figure 1A). Compounds DM58, DM54, and DM75, to which a mannoside moiety having a phenyl ring was introduced by Sonogashira coupling, in contrast, showed significant potency. The position of the phenyl ring appeared to be rather important with regard to modulating the activity of DM58 and which differs from DM54 only by the relative positioning of the phenyl ring (Figure 1B).

In conclusion, we report the biological evaluation and the identification of specific mannosides which appear to play an important role against the interaction between lectins and carbohydrate glycoconjugates present at the cell surface of MSC. The specific action of these mannosides revolves around their capacity to antagonize MT1-MMP-mediated events induced by ConA and that involve cell death signaling and activation of latent proMMP-2 in MSC. While novel approaches to the inhibition of MT1-MMP activity are explored (Arroyo et al. 2007), our observations may, thus, find broader therapeutic implications than only in MSC recruitment, cell surface glycoprotein-mediated cell–cell contacts and engraftment within a tumor environment. Increased expression of stem cell markers in malignant melanoma including cell surface glycoproteins such as the activated leukocyte adhesion molecule (CD166) and prominin-1 (CD133) have recently been revealed as potential new prognostic markers (Klein et al. 2007). Given the noncytotoxic effects of the mannosides tested in our study (Figure 2, see vehicle panels), it can now be envisioned to test those mannosides in vivo against experimentally implanted tumors. Based on our results, it is further tempting to suggest that the specific targeting of cell surface glycoproteins by those mannosides may, in part, be directed against MT1-MMP-mediated processes that regulate cancer cells survival, metastasis, and endothelial cell-mediated angiogenesis. As such, efficient delivery of those mannosides may achieve both direct tumor cell killing and indirect tumor cell killing via the destruction of tumor-associated endothelium or recruitment/engraftment of circulating cells.

Materials and methods

Materials

Sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON, Canada). Cell culture media were obtained from Life Technologies (Burlington, ON, Canada). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON, Canada). The enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). The polyclonal antibodies against MT1-MMP (AB815) and ERK were respectively from Chemicon (Temecula, CA) and from Santa Cruz Biotechnology (Santa Cruz, CA), while the polyclonal antibody against G6PT was generated against the 5-GYGYYRTVIFSAMFGGY-21 peptide derived from the human G6PT primary sequence (accession no. AAD19898) (Chen et al. 2002) at the Biotechnology Research Institute (Montreal, QC, Canada). All other reagents were from Sigma-Aldrich Canada.

Cell culture

Bone marrow-derived MSC were isolated from the whole femur and tibia bone marrow of C57BL/6 female mice, and cells were cultured and characterized as previously described (Meriane et al. 2006). Analysis by flow cytometry, performed at passage 14, revealed that MSC expressed CD44 yet were negative for CD45, CD31, KDR/flk1 (VEGF-R2), flt-4 (VEGF-R3), and Tie2 (angiopoietin receptor) (data not shown).

Hemagglutination inhibition assay

Hemagglutination assays were carried out in round-bottomed microtitre plates. A total volume of 100 μL was used in each well: 25 μL of ConA (0.1 mg/mL) was added to 25 μL aliquots of serial 2-fold dilutions of the different mannosides synthesized (0.5 mg/mL stock solutions) and 50 μL of 3% (v/v) rat erythrocyte suspension in HEPES buffer (0.1 M HEPES, 0.15 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$, pH 7.2). The microtitre plate with the 100 μL erythrocyte suspension, containing the serial double dilutions of the tested sugars, was incubated for 3 h at room temperature. The lowest concentration of the sugars required to completely inhibit red blood cells agglutination by ConA was determined visually (Dam et al. 1998).

Gelatin zymography

Gelatin zymography was used to assess the extent of latent and proMMP-2 and active MMP-2 activity. Briefly, an aliquot (20 μL) of the culture medium was subjected to SDS–PAGE in a gel containing 0.1 mg/mL gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled H_2O. Gels were further incubated at 37°C for 20 h in 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, 50 mM Tris–HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid, 30% methanol in H_2O. Gelatinolytic activity was detected as unstained bands on a blue background.

Fluorimetric caspase-3 activity assay

MSC were grown to about 80% confluence in 6-well dishes and treated for 16–18 h with ConA or CytoD either lacking, or in combination with, mannan, DM58, DM54, or DM75 mannosides. After treatment, cells were collected and washed in ice-cold PBS pH 7.0. Cells were lysed in Apo-Alert lysis buffer (Clontech, Palo Alto, CA) for 1 h at 4°C and the lysates were clarified by centrifugation at 16,000 × *g* for 20 min. Caspase-3 activity was determined by incubation with 50 μM caspase-3-specific fluorogenic peptide substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) in assay buffer [50 mM Hepes-NaOH (pH 7.4), 100 mM NaCl, 10% sucrose, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 5 mM DTT, and 1 mM EDTA] in 96-well plates. The release of AFC was monitored for at least 30 min at 37°C on a fluorescence plate reader (Molecular Dynamics) ($\lambda_{ex} = 400$ nm, $\lambda_{em} = 505$ nm).

Immunoblotting procedures

Proteins from control and treated MSC were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1000 dilution) in TBST containing 3% bovine serum albumin, followed by 1 h incubation with horseradish peroxidase-conjugated antirabbit IgG in TBST containing 5% nonfat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC, Canada).

Analysis of cell cycle by flow cytometry

Distribution of BMSC throughout the cell cycle was assessed by flow cytometry (Currie et al. 2007). Serum-fasting preparation was performed prior to analysis, and therefore the cell populations were synchronous. Cells were harvested by gentle scraping, pelleted by centrifugation, washed with ice-cold PBS/EDTA (5 mM), then resuspended in 1 volume PBS/EDTA and fixed with 100% ethanol overnight. Three volumes of staining solution, containing propidium iodine (PI, 50 μg/mL), and DNAse-free RNAse (20 μg/mL), were added. The fraction of the population in each phase of the cell cycle was determined as a function of the DNA content using a Becton Dickinson FACS Calibur flow cytometer equipped with CellQuest Pro software. In particular, the characteristics of cell distribution in the subG1 region were studied on the DNA histogram.

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Conflict of interest statement

None declared.

Abbreviations

BSA, bovine serum albumin; ConA, concanavalin-A; CytoD, cytochalasin-D; ECL, enhanced chemiluminescence; ECM, extracellular matrix; ER, endoplasmic reticulum; ERK, extracellular signal-regulated protein kinase; G6PT, glucose-6-phosphate transporter; MMP, matrix metalloproteinase; MSC, mesenchymal stromal cells; MT1-MMP, membrane type-1 MMP; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate.

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