Gold Glyconanoparticles as New Tools in Antiadhesive Therapy

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*Gold glyconanoparticles (GNPs) have been prepared as new multivalent tools that mimic glycosphingolipids on the cell surface. GNPs are highly soluble under physiological conditions, stable against enzymatic degradation and nontoxic. Thereby GNPs open up a novel promising multivalent platform for biological applications. It has recently been demonstrated that specific tumor-associated carbohydrate antigens (glycosphingolipids and glycoproteins) are involved in the initial step of tumor spreading. A mouse melanoma model was selected to test glyconanoparticles as possible inhibitors of experimental lung metastasis. A carbohydrate – carbohydrate interaction is proposed as the first recognition step for this process. Glyconanoparticles presenting lactose (*lacto-*GNPs) have been used successfully to significantly reduce the progression of experimental metastasis. This result shows for the first time a clear biological effect of* lacto-*GNPs, demonstrating the potential application of this glyconanotechnology in biological processes.*

Introduction

The surface of mammalian cells is covered with a dense coat of carbohydrates named glycocalyx. There is evidence that this glycocalyx is critically involved in cell-adhesion and cell-recognition processes. Nowadays, the important role of carbohydrates in a broad spectrum of physiological and pathological processes, including metastasis,[1, 2] inflammation,[3] and infection,[4] is well established. All these processes imply a protein – carbohydrate interaction, but there is now clear evidence that carbohydrate – carbohydrate interactions between glycosphingolipid (GSL) clusters are also involved in these processes.[5, 6] Carbohydrate interactions are characterized by dependence on divalent cations and low affinity binding, which is compensated for in Nature by multivalent presentation of the ligands. Different approaches have been developed to study carbohydrate interactions, all of which are based on a multivalent presentation of carbohydrate ligands.[7–9] Chemical approaches to carbohydrate-based therapeutics are also emerging.[10–12] We have recently developed a new multivalent model system consisting of a metallic core to which self-assembled monolayers of glycoconjugate ligands are covalently linked.[13] These so-called glyconanoparticles (GNPs) are highly soluble in water and stable for months under physiological conditions without flocculation. Globular and with a chemically well-defined structure, they provide a glycocalyx-like surface that mimics the presentation of glycosphingolipid clusters at the cell surface. Therefore, GNPs are adequate tools for basic studies in carbohydrate interactions and for intervention in cell – cell adhesion processes.[13, 14] In this report, we illustrate the first application and validation of GNPs as antiadhesion tools against metastasis progression.

In metastasis, tumoral cells detach from the primary tumor and travel through the lymphoid and blood vessels until they arrive at a specific target location. One of the critical steps in metastasis is the adhesion of tumor cells to the vascular endothelium. After adhesion, tumor cells transmigrate and create new tumor foci (seed and soil theory).[15] Metastasis is the origin of the bad prognosis of most cancers. The majority of tumor cells, independent of their pathophysiological origin, display an aberrant glycosylation pattern on their surfaces, and this common feature is thus an interesting target for the development of new therapies.[16, 17] Interactions between tumor-associated carbohydrate antigens and epithelial cell selectins promote tumor cell metastasis.[18] In addition to this

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mechanism, carbohydrate–carbohydrate interactions between GSLs expressed on the tumor and endothelial cell surfaces also seem to be involved in the critical adhesion step.[19] Therefore, inhibition of this step by glyconanoparticles that present carbohydrate antigens expressed either on tumor or endothelial cells might provide effective antiadhesion therapy and thus negatively influence tumor progression.

A murine melanoma cell line (B16) that generates aggressive metastasis in lungs has been used as the model system to study metastasis and tumor progression.[20] In this model, a direct correlation between cell-surface expression of the ganglioside GM3 (NeuNAc2α3Galβ4GlcβCer) and aggressiveness has been established.[21, 22] Apart from that, the main glycoconjugates presented on the surface of the lung endothelium are lactosylceramide (Galβ4Glcβ-Cer), Gg3 (GalNAcβ4Galβ4GlcβCer), and Gb4 (GalNAcβ3Galα4Galβ4GlcβCer). Hakomori et al.[23] have proposed a carbohydrate–carbohydrate interaction between GM3 of B16 cells and Gg3 or lactosylceramide of endothelium cells as the first and critical step of tumor cell adhesion to the endothelium before transmigration. This step can be envisaged as a potential target to inhibit cancer progression during metastasis. Hakomori et al.[22] used a polyvalent carbohydrate model system based on liposomes incorporating Gg3 or lactosylceramide, which is able to inhibit the adhesion of Bl6B26 murine melanoma cells to lung endothelium.

Based on this precedent, we explore here the ex vivo metastasis inhibitor and antiadhesion potential of GNPs as novel polyvalent system by using the murine melanoma model.

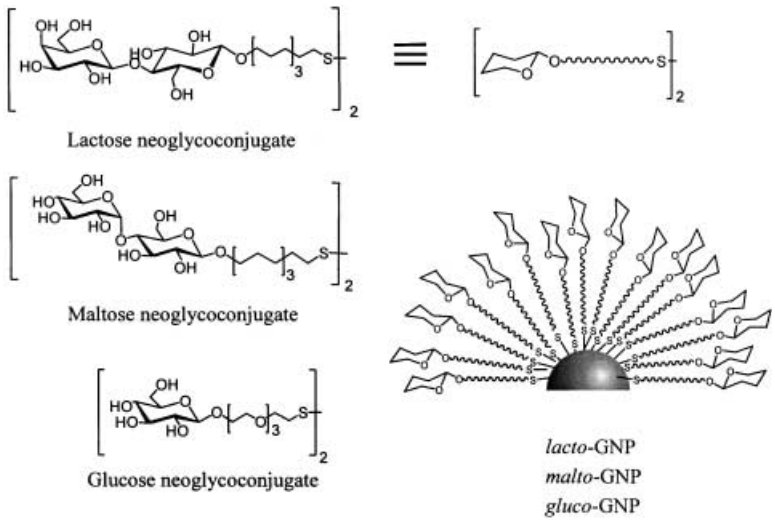

Figure 1. *Neoglycoconjugates and glyconanoparticles used in this study.*

Results and Discussion

Glyconanoparticle technology

A new technology has been developed to prepare gold glyconanoparticles (GNPs) presenting carbohydrates in a globular and polyvalent configuration at their surface (Figure 1). This technology allows the preparation of water-soluble and exceptionally small nanoclusters (diameter below 2 nm) functionalized with specific carbohydrate antigens. The particles are prepared by in situ reduction of a gold salt in the presence of an excess of the corresponding thiol-derivatized neoglycoconjugate.[13b] Manipulation of the ratio of gold salt to organic ligand permits control of the nanoparticle's size and polyvalence.

GNPs present some advantages over other currently available polyvalent systems incorporating carbohydrates, such as liposomes: 1) strict control of ligand numbers and nanoparticle size, 2) higher degree of multivalence and easy chemical characterization, 3) high storage stability, and 4) high biological stability against enzyme degradation.

Based on the involvement in cell adhesion of the tumor associated antigens,[16, 17] lactosylceramide and lactoneotetraosylceramide (nLc4Cer), we have prepared nanoparticles bearing 70 lactose molecules (*lacto*-GNPs) to be tested as a potential inhibitor of the binding of melanoma cells to endothelium cells. Glyconanoparticles functionalized with the disaccharide maltose (Glcα4Glc, *malto*-GNPs) and the monosaccharide glucose (*gluco*-GNPs), which are not involved in this adhesion process, have also been prepared as control systems (Figure 1).

The application of the GNPs to biological models needs to be preceded by cytotoxicity studies. Incubation of B16F10 cells with *lacto*-GNPs or *gluco*-GNPs for 24 h did not show any significant negative effect on cell survival when compared with untreated B16F10 cells. In contrast, *malto*-GNPs demonstrated clear deleterious effects on cellular viability, probably due to nonspecific interactions with cell membranes (Figure 2). In addition, after the above-mentioned treatments with *lacto*- and *gluco*-GNPs (even at 90 μM concentration, data not shown), B16F10 cells were recovered, washed with phosphate buffered saline

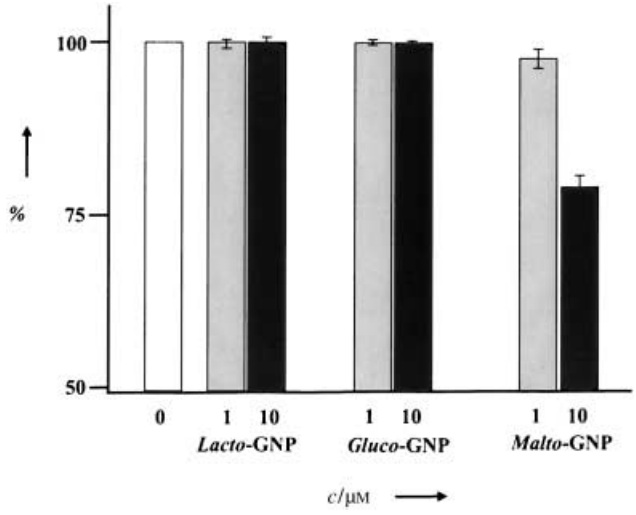

Figure 2. *Evaluation of the cytotoxicity of GNPs. Percentage of murine melanoma B16F10 cell line viability after 24 h incubation period in the absence and presence of 1 and 10 μM concentrations of* lacto-, gluco- *or* malto-*GNPs.*

(PBS) and seeded again in fresh medium. No significant alterations in their morphology or growth kinetics could be observed in relation to the untreated cells (data not shown). Similar results were also obtained when COS-1, embryonic F9 or NIH-3T3 cells were evaluated for GNP-induced cytotoxicity. *Lacto*- and *gluco*-GNPs can therefore be considered as nontoxic model systems to be used in biological studies. On the other hand, *malto*-GNPs induced negative effects when the treatment was prolonged for 6 – 24 h, although they did not show effects on cell cultures at short incubation times (0 – 120 min). For this reason, *gluco*-GNPs were used as negative control in the ex vivo experiments.

We have evaluated the effect of *lacto*-GNPs on the metastatic potential of the B16F10 melanoma cell line to induce tumoral foci in lung upon intravenous (i.v.) inoculation in C57/Bl6 mice. An ex vivo experiment with *lacto*-GNPs and the B16F10 melanoma cell line has been designed. The experimental design (Figure 3) included a comparative in vivo evaluation of the

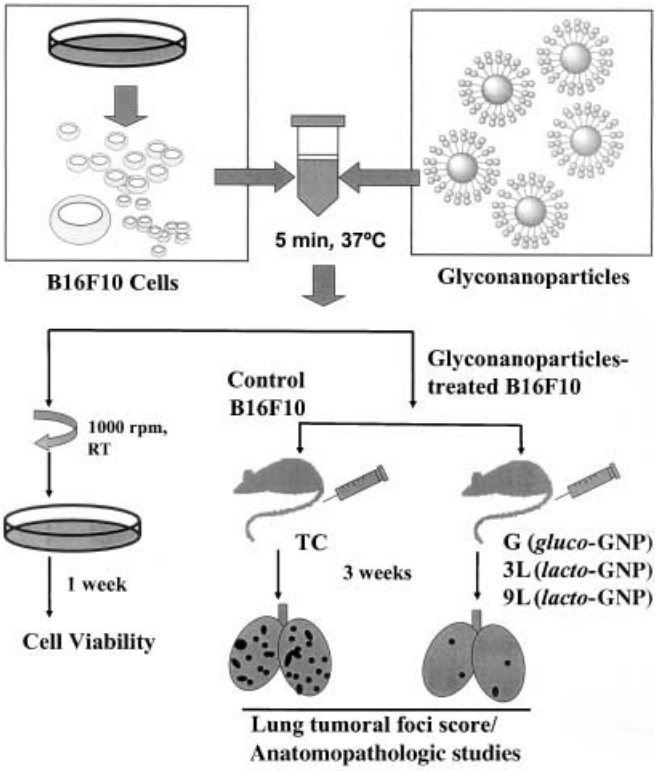

Figure 3. *Schematic representation of the experimental design for the evaluation of the antimetastatic potential of* lacto-*GNPs. Control group C (mice inoculated only with* lacto-*GNPs in Hank's solution) is not represented.*

efficiency of the B16F10 cell line to generate specific lung metastases, either with or without a previous short incubation of the cells with *lacto*-GNPs or *gluco*-GNPs. Five groups of mice were used for the experiment. One group (C) was tail-vein inoculated with *lacto*-GNPs (90 μM) in 200 μL of Hank's solution as in vivo cytotoxic control. A second group (TC, a positive control) was injected with a single dose of B16F10 melanoma cells (10^5 cells in 200 μL of Hank's solution). The other three groups were treated with single doses of B16F10 melanoma cells (10^5 cells in 200 μL of Hank's solution) preincubated for 5 min at 37 °C with 90 μM of the control *gluco*-GNPs (G-GNPs) or with 30 or 90 μM of *lacto*-GNPs (3L-GNPs and 9L-GNPs, respectively). No adverse effect was observed in the animals upon i.v. injection of the GNP-containing suspension (group C). As a control experiment, an aliquot of the B16F10 cell suspensions preincubated with the GNPs was processed for cell recovery (sedimentation at 1000 rpm and room temperature, followed by a washing step with PBS) and kept in culture for one additional week, monitoring their growth kinetics to detect any potential deleterious effects induced by the brief preincubation with GNPs. No deleterious effect due to preincubation with *gluco*- or *lacto*-GNPs was observed (data not shown). The experimental metastatic process was allowed to develop for three weeks and the animals were then sacrificed, necropsy samples of different organs taken and both lungs evaluated under a microscope for tumor foci analysis.

All the animals included in the experiment were carefully inspected during the whole period of time. Some of the animals presented bristled hair in the first days but no other special behavioral or pathological effects were noticed. Samples were taken from several organs for histological analyses that did not reveal any significant alteration. These data clearly demonstrate that i.v. inoculation of mice with *lacto*- or *gluco*-GNPs (up to 90 μM) does not promote any relevant toxic effect in live animals or any histopathological manifestation.

Direct visual inspection of lungs obtained from animals inoculated with B16F10 cells pretreated with *lacto*-GNPs showed a strong protective effect against lung metastasis in contrast to those obtained from animals primed with B16F10 cells or B16F10 cells pretreated with *gluco*-GNPs (Figures 4 and 5). Lung metastases foci scoring indicated that the experimental group inoculated with B16F10 cell line (TC group) and with B16F10 cell line preincubated with *gluco*-GNPs (G-GNPs group) at concentrations of 90 μM had developed a high number (98 ± 40 per lung) of new tumor foci (Figures 4 and 5 B). However, the group

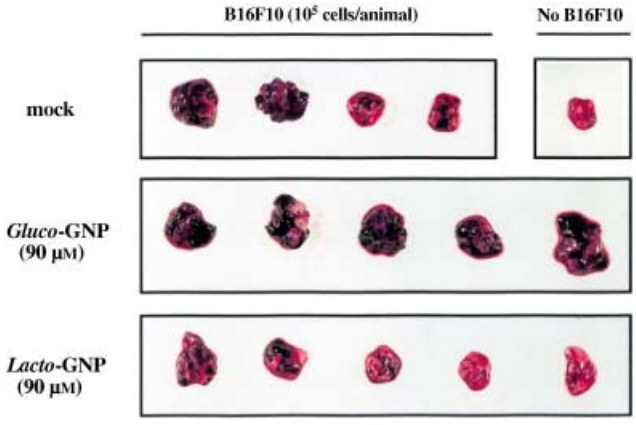

Figure 4. *Specific antimetastatic effect of* lacto-*GNPs on the B16F10-dependent development of lung tumoral foci. A representative picture of lungs corresponding to animals included in each group in comparison with the lungs obtained from a control animal not injected with B16F10 cells.*

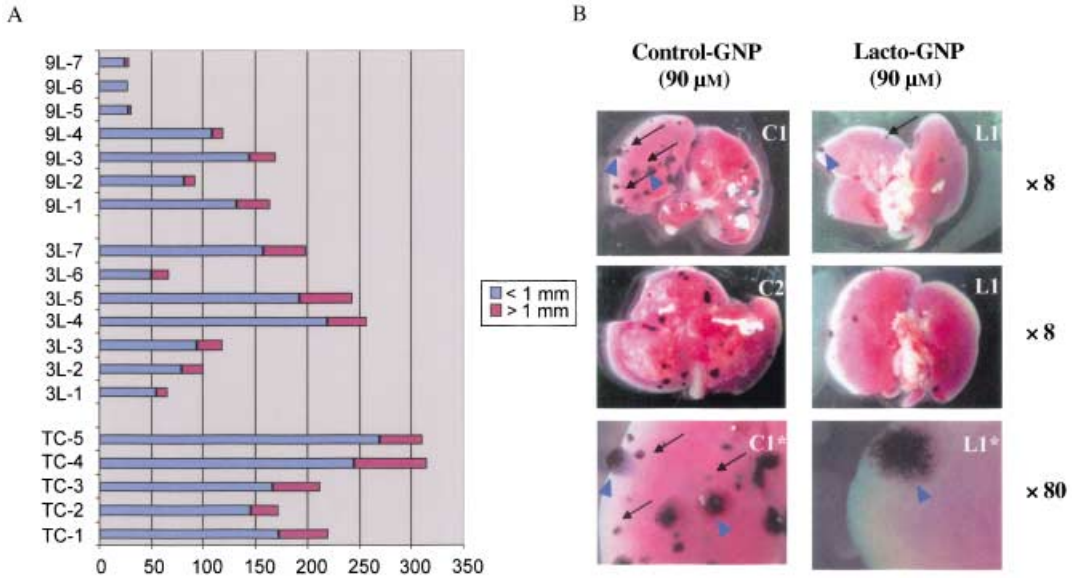

Figure 5. *Quantitive analysis of the antimetastatic effect of* lacto*-GNPs on the B16F10-dependent development of lung tumoral foci. A) Individual scoring of tumoral foci for five animals corresponding to the TC control group [TC-1 to TC-5] and seven animals treated with 30 μM [3L-1 to 3L-7] or 90 μM [9L-1 to 9L-7] solution of* lacto*-GNPs. B) Pictures of the lungs corresponding to TC-1, TC-2, 9L-6, and 9L-7 mice at two different magnifications (× 8, × 80). Black arrows indicate the small foci (< 1 mm) and blue arrows denote the presence of large ones (> 1 mm).*

inoculated with B16F10 cells preincubated with *lacto*-GNPs (L-GNP group) at two different concentrations (30 and 90 μM) presented a moderate inhibition of metastasis process in the first case, that is close to 70% inhibition media (92 – 53% range variation (Figures 4 and 5) in the case of the higher concentration. The inhibitory potential of the *lacto*-GNPs at the lower concentration is more evident on smaller metastatic foci (almost 30% reduction on foci < 1 mm) than on the larger (0 – 2% inhibition on foci > 1 mm), whereas at higher concentrations both types of foci seem to be affected (Figure 5 A). The dispersion and inter-animal variation was quite high (Figure 5 A and B), but clearly some animals (5 – 7) were almost totally protected against the formation of tumoral foci. In each experimental group, three animals were kept alive in order to test whether the remaining tumors (not eliminated by the GNP-pretreatment) were aggressive enough to kill the animal, or whether the GNP-pretreatment of the B16F10 murine melanoma cells could induce an in vivo selection for some kind of nonaggressive clonal tumoral cells. All these animals died; this indicates that the remaining tumoral foci are fully competent. This experiment clearly shows both a selectivity and a dose-dependent effect of *lacto*-GNPs in the experimental inhibition of the aggressive lung metastasis promoted by B16F10 melanoma cells.

Further in vitro experiments have been carried out to dissect the molecular mechanisms by which nanoparticles may inhibit metastasis. First of all, an experiment to evaluate the antiadhesion ability of this new model system was designed. B16F10 cells were incubated (5 min at 37 °C) with a 5 μM solution of *gluco*-GNPs, *malto*-GNPs, or *lacto*-GNPs, and the cellular suspensions were seeded into plastic cell-culture wells. Aliquots were removed at different times, and the number and viability of cells attached to the plastic wells were determined by using the classical 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[24] Cells preincubated with control-GNPs (*gluco*-GNPs and *malto*-GNPs) and those not treated with GNPs adhere to the plastic cell culture surface with normal and comparable kinetics. Those cells pretreated with *lacto*-GNPs presented, however, a decreased efficiency in seeding that became drastic over time (Figure 6 A). This result indicates that in the initial reversible phase of cell adhesion *lacto*-GNPs are unable to inhibit the pre-adhesion cell contacts with the culture surface (1 – 20 min). At a later stage (after 20 min) however, *lacto*-GNPs seem to block the extensive cell-matrix contacts needed for cellular spreading and proliferation, finally provoking cell-detachment from the matrix (Figure 6 A). Two possible nonexcluding reasons can be proposed for this finding: *Lacto*-GNPs could specifically interact with proteins of the melanoma cell responsible for establishing strong contacts after the first reversible phase.[22] Alternatively, *lacto*-GNPs might activate cell-signaling pathways that will dictate the later detachment (20 – 60 min) from the matrix of the preadhered B16F10 cells.[19] Both alternatives are currently being studied while working on specific cell-surface extracellular matrixes that mimic natural templates. In any case, the results obtained here indicate the ability of *lacto*-GNPs to selectively interact with the B16F10 cells, while this interaction is not mediated by the control GNPs (*gluco*-GNPs and *malto*-GNPs).

The potential of *lacto*-GNPs to interfere with the adhesion of the B16F10 cells to endothelial cells was also evaluated (Figure 6 B). B16F10 cells, previously labeled with the BCECF-AM fluorescent reagent,[25] were preincubated with different concentrations of *lacto*- and *gluco*-GNPs and seeded onto pre-established monolayers of the bEnd.3 murine endothelial cell line. After 1 h of co-culture, which allowed functional interactions between both cell types, the culture medium and the

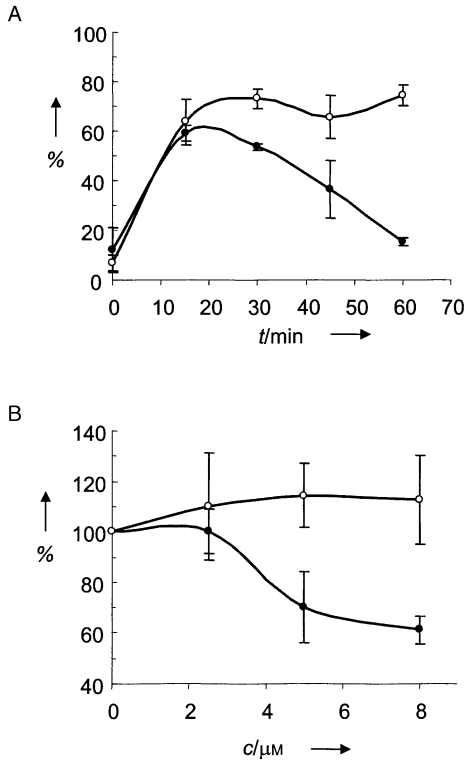

Figure 6. *Selective interference of lacto-GNPs on adhesion of B16F10 cells. A) Percentage of adhered B16F10 mouse melanoma cells to plastic well plate, preincubated with* lacto-*GNPs (●) or* gluco-*GNPs (○) at different times. B) Percentage of B16F10 cells adhered to a monolayer of the bEnd.3 murine endothelial cell line preincubated with* lacto-*GNPs (●) or* gluco-*GNPs (○) at different concentrations.*

unattached cells were removed and replenished with fresh complete medium to continue the culture for an additional 24 h. Cultures were then harvested by careful trypsinization to single-cell level, and the presence of viable labeled B16F10 cells was quantified by cell cytometry. Clearly, preincubation of B16F10 with the control GNPs has no effect on the adhesion of tumor cells to endothelial cells. However, *lacto*-GNPs induce a specific reduction of the capacity of the cells to permanently adhere to an endothelial cell monolayer (Figure 6 B); this indicates once more the specific binding of the *lacto*-GNPs to tumoral cells.

In this exploratory study, we have evaluated the potential of GNPs presenting the lactose antigen in a globular and polyvalent configuration (*lacto*-GNPs) as new antiadhesion tools against metastasis progression. Short ex vivo preincubation of tumoral cells with *lacto*-GNPs is enough to substantially inhibit lung metastasis (up to 70 %) in a well-defined in vivo tumor metastasis model, although the process is not capable of eradicating the phenomenon. This is an expected result because it has been demonstrated that other adhesion factors, such as integrins, play a role.[26] The obtained results, however, demonstrate both the specificity of the *lacto*-GNPs in the selected model and their antiadhesion properties, which can be improved in later generations of GNPs.

The GNPs developed and used in this study can intervene in cell – cell adhesion processes mediated by both carbohydrate – lectin and/or carbohydrate – carbohydrate interactions. This finding opens a new avenue for the design and preparation of GNPs bearing carbohydrate antigens for a plethora of applications in other antiadhesion therapies (inflammation, infection, etc.) as well as in microarray technology and molecular diagnostics.

In the last decade, advances have led to the development of metallic nano-bioconjugates based on proteins and nucleic acids for applications such as biosensors, biomaterials, and biotechnological tools.[27–34] We have developed a simple methodology to prepare, for the first time, water-soluble gold nanoclusters functionalized with carbohydrate antigens as tools for basic studies on specific carbohydrate recognition.[13, 14] The globular shape and the multivalent display of the oligosaccharides at their surface convert GNPs into powerful tools to overcome the low-affinity binding of monomeric oligosaccharide to protein or carbohydrate receptors. In addition, a great diversity of glyconanoparticles with varying carbohydrate antigens and also differing in carbohydrate density can be prepared by this methodology, providing a controlled model to study the influence of carbohydrate presentation and density on their recognition events.[13b] The methodology includes the preparation of hybrid GNPs incorporating both carbohydrates and other molecules, such as fluorescence probes, peptides, biotin, etc. Furthermore, the preparation of *glyco* quantum dots as well as GNPs with magnetic properties can envisage the potential of this novel technology (unpublished results).

Conclusion

Multivalent binding in carbohydrate-mediated interactions is a ubiquitous phenomenon in nature. The development of carbohydrate-based multivalent systems has profoundly contributed to the understanding of carbohydrate-mediated biological processes. We have developed a simple and versatile strategy for tailoring functionalized gold nanoclusters (glyconanoparticles, GNPs) with multivalent carbohydrate display and globular shape.[13] The GNPs complement other currently available multivalent systems incorporating carbohydrates and present some additional advantages such as: 1) high polyvalence with control over ligand number and nanoparticle size, 2) water solubility, and 3) high storage stability as well as resistance to enzyme degradation. GNPs with biologically significant oligosaccharides and with differing carbohydrate densities have been prepared to intervene in cell adhesion processes. This paper describes the application of this multivalent system as an antiadhesive tool in tumoral metastasis progression in vivo; it shows the potentiality of this glyconanotechnology for use in other antiadhesion therapies. The glyconanoparticle principle described here has the potential to integrate all the current knowledge and applications on processes that involve a carbohydrate molecule (inflammation, viral, bacterial, and toxin infection etc.) as well as the recent developments in bionanotechnology. The "proof of principle" established here for the possible biological applications of GNPs prompts us to anticipate an important advance in a field that may be specifically named glyconanotechnology and that will complement current gene-oriented nanotechnology.

Experimental Section

Glyconanoparticle technology: The synthesis of the glyconanoparticles was carried out by using the methodology previously developed in our laboratory.[13] The glyconanoparticles *lacto*-GNP, *malto*-GNP, and *gluco*-GNP were obtained by adding a solution of the corresponding neoglycoconjugate in methanol to an aqueous solution of tetrachloroauric acid ($HAuCl_4$) (Figure 1). By reduction of the mixture with $NaBH_4$ a yellow to dark suspension formed immediately. The prepared glyconanoparticles were purified by washing with methanol, NANOPURE water and centrifugal filtering (CENTRIPLUS MW 30000, 1 h, 3000 g). The process was repeated until the nanoparticles were free of salts and starting material (absence of signals due to neoglycoconjugates and Na^+ in the ^1H and ^{23}Na NMR). The residue in the CENTRIPLUS filter was dissolved in NANOPURE water and lyophilized. For biological experiments the GNPs were dialyzed three times at 4 °C against PBS (3 L) in 10 K Slide-A-Lyzer® dialysis cassettes (Cat No.66425, Pierce, Rockford, IL). To remove undissolved particles and to ensure sterile conditions, the dialyzed GNP-containing suspensions were filtered through low protein binding filters (0.22 μm pore size, Millipore, Bedford, MA). All purified GNP solutions were stored at room temperature and protected from light. The nanoparticles were characterized by NMR, IR, UV spectroscopy, and transmission electron microscopy (TEM). The nanoparticles molecular formula was calculated based on the average core diameter obtained by TEM[35] and confirmed by elemental analysis. A mean diameter of 1.8 nm was found for the gold core of *lacto*- and *gluco*-GNPs, which corresponds to an average number of 70 sugar molecules and 201 gold atoms (76 kDa MW) per particle. The gold atoms for the *malto*-GNP core were less than 79. This small core ($\leq$1 nm) does not allow the exact determination of the gold atoms.

Cell lines and culture conditions: The B16F10 mouse melanoma cell line was obtained from the American Type Culture Collection (Manassas, VA) and was maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc), supplemented with 10% fetal calf serum (FCS; Gibco-BRL; Gaithersburg, MD), 1% L-glutamine (Merck, Darmstadt, Germany), streptomycin (0.1 mg mL^{-1}, Sigma, St. Louis, MO), penicillin (100 U mL^{-1}, Sigma), 1% sodium-pyruvate (Sigma), 1% unessential amino acids (Bio-Whittaker, Walkersville, MD) and 50 μM β-mercaptoethanol (Sigma). The murine endothelial cell line bEnd.3 was kindly provided by Dr. Rodriguez-Frade (DIO, CNB, Madrid) and maintained in equivalent conditions to those described for the B16F10 cell line. NIH-3T3, F9 and COS-1 cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc), supplemented with 10% fetal calf serum (FCS; Gibco-BRL), 2 mM L-glutamine (Merck, Darmstadt, Germany), streptomycin (0.1 mg mL^{-1}, Sigma) and penicillin (100 U mL^{-1}, Sigma). All cell cultures were grown in a humidified 37 °C incubator with 5% CO_2 and periodically tested for being mycoplasma-free by using specific commercial kits (Gen-Probe, San Diego, CA).

In vitro cell growth monitoring and cytotoxicity tests: Cell cultures were routinely monitored by cell scoring by using a haemocytometer chamber after careful trypsinization of the adherent cell monolayer by using the trypan-blue vital dye. Cellular proliferation was determined by using the (MTT) colorimetric assay as described and by direct cell counting.[24] Briefly, 10^5 cells/well were seeded into 96-well plates in 100 μL complete medium and incubated for the indicated time periods, in the absence or presence of 1 and 10 μM Hank's solution of *lacto*-, *gluco*- or *malto*-GNPs. After a 24 h incubation period, viability of the culture cells was evaluated by using the MTT method as follows: a solution (10 μL) of tetrazolium salt (MTT, 25 mg mL^{-1} in PBS) was then added to each well and incubated at 37 °C for 4 h. Under these conditions, MTT is reduced by living cells into an insoluble blue formazan product that is collected by centrifugation and solubilized by the addition of DMSO (100 μL) with vigorous shaking. Plates are then read with a multi-well scanning spectrometer at 540 nm.

In vitro cell adhesion: B16F10 mouse melanoma cells, preincubated with different GNPs (5 min at 37 °C, in complete DMEM medium and at the indicated concentration of GNPs), were seeded (1x10^5/100 μL/well) into 96-well plates. At the indicated culture times (0–60 min) the culture medium was removed and exchanged for fresh complete medium. Culture of the adhered B16F10 cells was maintained for an additional 6–12 h and viable cells remaining in the cultures were scored by the previously described MTT method.

B16F10 cells (10^5/well in 50 μL of complete medium), previously labeled with BCECF-AM (Molecular Probes Inc, Eugene, OR) and treated as above with the GNP suspensions, were evaluated for their adhesion properties to a monolayer of the bEnd.3 murine endothelial cell line. Labeled B16F10 cells were co-cultured onto the bEnd.3 monolayer for 1 hour. Thereafter, culture medium with the unattached cells was discarded and replaced by fresh medium to maintain the culture for additional 24 h. Cultures were then harvested and exhaustively trypsinized to single-cell dispersion. Quantification of labeled and viable B16F10 cells was carried out by direct fluorescence in an EPICS XL-MCL cytometer (Coulter Corp., Miami, FL).

In vivo tumor model for lung metastasis: C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were bred in microisolator cages in the barrier facility of the Centro Nacional de Biotecnología under standard pathogen-free protocols. All procedures were designed according to recommendations in *The guide for care and use of laboratory animals*, National Research Council, National Academic Press, 1996.

In vivo tumor formation was induced by i.v. injection of B16F10 cells (1×10^5) in 8–12 week-old female C57 BL/6 mice in 200 μL of Hank's solution (Gibco-BRL), inoculated into the tail vein. Three to four weeks after inoculation and always before pathological symptoms appeared the animals were killed and the lungs were removed for careful inspection and tumoral foci scored after fixation in Tell-eyeniczky's solution (44% ethanol, 31% acetic acid and 2.3% formaldehyde). An extensive anatomopathological analysis of the sacrificed mice was carried out when indicated.

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