Gangliosides are one of the immunosuppressive molecules released by tumors to their microenvironment. These glycosphingolipids are differentially distributed in tumor vs. normal tissues, and gangliosides containing an N-glycolylated variant of sialic acid have been found to be preferentially tumor-associated in humans. One such ganglioside is N-glycolyl GM3 (NGcGM3), which has become an attractive target for antigen-specific antitumor therapy. Since the differential expression of this ganglioside in advanced human tumors contrasts with the trend towards reduced immunogenicity typical of cancer cells, it has been presumed that this molecule plays an important role in tumor biology. Therefore, we have studied the relevance of NGcGM3 for tumoral development and, specially, its influence on the immune response. Our results show that NGcGM3 contributes to tumoral progression by modifying helper T lymphocyte function. This ganglioside reduces the expression of CD4 in T lymphocytes, inserting into the plasma membrane of these cells. Furthermore, it affects cell proliferation and promotes a differentiation towards an anti-inflammatory cytokine secretion profile in CD4+CD25− T lymphocytes. The latter effect is not caused by increased suppression mediated by naturally occurring regulatory T lymphocytes. Additionally, NGcGM3 also affects the differentiation and maturation of dendritic cells.

Introduction

The identification of molecules with altered expression patterns in tumoral cells when compared to normal tissues is the main strategy for the development of active or passive antigen-specific antitumor immunotherapies. The targets selected for this strategy should ideally fulfill an important role in tumor biology, allowing the induction of a specific antitumoral immune response that interferes with key biological events associated to tumoral progression.

In spite of the implementation of multiple alternatives for the generation of an effective immune response against tumors, cancer remains a serious public health problem worldwide with a high mortality rate. The immunotherapies used so far have not been sufficiently effective, due among other factors to the nature of the interactions between the tumor and the immune system of the host. Proof argue for the existence of an immunological surveillance system against neoplasias, which involves different effector mechanisms of the immune system that prevent the appearance of tumors or limit their progression once they are established. However, the selective pressure exerted by the immune system favors the selection of resistant tumor variants. This phenomenon, known as tumor editing, is facilitated by the high mutation rates that characterize cancer cells [1], and also leads to the activation of immunosuppressive mechanisms in tumors. In this context, it has been shown that tumor cells can release molecules to their microenvironment that modulate antigen presentation and have a negative impact on lymphocyte proliferation, affecting the activity of immune effector cells [2].

It has also been shown that tumor progression is correlated with an increased frequency of cells with suppressive properties [3]. Gangliosides are a well-known example of immunosuppressive molecules [4]. They constitute the most variable glycosphingolipid group, and are characterized by the presence of at least one sialic acid moiety in their structure. Gangliosides are expressed on the plasma membrane of vertebrate cells, where they constitute an essential component of the so-called lipid micro-domains, which are cholesterol-rich structures that function as anchoring points for a large variety of proteins involved in cellular signal transduction.

The distribution of gangliosides among different tissues is highly heterogeneous and depends on the ex-tent of cellular differentiation. There are changes in ganglioside composition in the plasma membrane of cancer cells, leading to the emergence of tumor-associated antigens [5]. That is the case of the N-glycolylated variant of sialic acid which, although widely distributed on different mammalian species, are almost absent in normal human tissues [6]. This scarcity is caused by the absence in humans of a functional gene for cytidine monophosphate-N-acetyl sialic acid hydroxylase, the enzyme that catalyzes the N-glycolylation of N-acetyl sialic acid [7].

It has been shown that dietary intake and latter incorporation of this modified variant into the cellular sialic acid pool account for the very low levels of N-glycolyl gangliosides detectable in normal human cells [8]. In cancer cells, however, the incorporation of this precursor is favored due to the increased expression of sialic acid transporters in the hypoxic environment of the tumor, leading therefore to increased levels of N-glycolyl gangliosides [9]. This phenomenon has turned these molecules into an attractive target for antigen-specific antitumoral therapy; and specially so for the N-glycolyl GM3 ganglioside (NGeGM3), which has been detected in human tumor cells such as those from infiltrating ductal carcinoma of the breast [10] and melanoma [11].

The expression of this ganglioside in advanced tumors contrasts with the fact that cancer cells down regulate those molecules that can become a target for

immune surveillance systems. In addition, little is known about the role of NGcGM3 in tumor biology and its interaction with cells from the immune system. These gaps in the existing knowledge about this system limit the design of more effective antitumoral therapies targeting NGcGM3, and restrict our understanding of the mechanisms of action for vaccine preparations currently under study.

Results

Influence of NGcGM3 on tumor growth in vivo

The murine myeloma X63, a cell line in which more than 85% of the total ganglioside content is formed by NGcGM3, was selected as a model for studying the influence of this molecule on tumor growth in vivo. X63 cells were cultured in the presence or absence of D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), a chemical compound that inhibits the activity of glucosylceramide synthetase. This procedure decreased by more than 60% the NGcGM3 contents of treated myeloma cells. Cell variants with varying ganglioside contents were then inoculated into Balb/c mice, followed by an evaluation of tumoral growth. The percentage of tumor-free animals was significantly higher in the group inoculated with cells expressing lower amounts of membrane-bound NGcGM3, and the average tumor diameter for this group was also lower [12]. The mutual influence between two X63 tumors with normal or reduced ganglioside expression levels, inoculated into the opposite sides of the same individual, was also evaluated. In this case the co-inoculation of both variants resulted in decreased growth for the variant expressing higher amounts of ganglioside, suggesting the successful induction of an effector immune response against the D-PDMP-treated line that was in turn effective against the normal myeloma. This result led to the study of the link between the effect of NGcGM3 in tumoral progression and the possibility of modifying the functional activity of the immune system.

Considering the importance of CD4+ T helper lymphocytes in the induction of a specific immune response, it was decided to evaluate whether the influence of NGcGM3 on tumoral progression was associated to its interaction with this cell population. Balb/c mice inoculated with the cell variants of X63 (pretreated or not with D-PDMP) were treated with a murine CD4+ T lymphocytes. Additionally, there were differences bet-

NGcGM3 ganglioside on the functional properties of CD4+ Th lymphocytes.

Effect of NGcGM3 on CD4+ Th lymphocytes

Highly purified NGcGM3 was used to determine the degree of negative regulation this molecule could exert over the expression of CD4 in murine and human lymphocytes. The results indicated a significant, dose-dependent decrease in CD4 expression caused by the ganglioside. The expression of other molecules relevant for lymphocyte function, such as CD3 and CD8, was not affected by this treatment [12].

The effect of the ganglioside on CD4 expression was reversible, since human lymphocytes pre-incubated with NGcGM3 and then cultured in its absence recovered CD4 expression levels up to 80% of their original values. The recovery was mediated by de novo synthesis, as evidenced when lymphocytes cultured in the presence of cycloheximide (an inhibitor of protein synthesis) failed to recover pre-treatment CD4 levels [12]. The sensitivity of the lymphocytes to NGcGM3-mediated CD4 down-regulation was unaffected after full recovery of CD4 expression.

In order to evaluate whether NGcGM3 was inserted into the plasma membrane of T lymphocytes, cells obtained from Balb/c lymph nodes and human peripheral blood mononuclear cells were incubated with different ganglioside concentrations. This treatment increased the levels of plasma membrane-associated NGcGM3 for both cell populations, in a dose-dependent manner [12]. The assay showed a statistically significant negative correlation between the phenomena of NGcGM3 membrane insertion and CD4 down-regulation in both murine and human Th lymphocytes (Figure 1b).

Mature non-activated and activated, as well as naturally occurring regulatory T lymphocytes were also used for studying the effect of the NGcGM3 ganglioside on CD4 expression. According to the results, the most pronounced down-regulation of CD4 mediated by NGcGM3 takes place in non-activated lymphocytes, with a less pronounced and similar effect for both activated and naturally occurring regulatory lymphocytes [13]. This indicates that the influence of the ganglioside on CD4 is independent from the expression of CD25, the receptor for interleukin 2 (IL2); in fact, the membrane levels of this molecule are not affected by NGcGM3.

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Influence of NGcGM3 on dendritic cell function

Based on the results outlined above and taking into account the role played by DC in the development of antigen-specific immune responses, it was decided to evaluate if NGcGM3 had any influence on DC function in the context of their interaction with Th lymphocytes, as well as any direct effects of the ganglioside on the differentiation and maturation of DC.

First, the impact of NGcGM3 on the proliferation of Th lymphocytes co-cultured with DC and stimulated with an anti-CD3 mAb was evaluated. The ganglioside decreased lymphocyte proliferation in a dose-dependent fashion for as much as 70% (Figure 2a). The remaining proliferative activity was accompanied by a switch of the cytokine secretion profile of these lymphocytes [13]. The presence of NGcGM3 during cell culture increased three-fold and two-fold the frequency of IL4- and IL10-secreting cells respectively, without increasing the percentage of interferon (IFN) gamma-producing lymphocytes (Figure 2b). This finding suggested that NGcGM3 not only changes the proliferation of Th cells, but also promotes the appearance of an anti-inflammatory cytokine secretion profile.

The evaluation of the effect of NGcGM3 on the differentiation of DC from their bone marrow precursors was performed with an in vitro differentiation assay in the presence or absence of the ganglioside. Dendropoiesis was decreased by NGcGM3, as evidenced by a significant reduction in the number of DC recovered from each culture well [13]. There was detectable IL10 mRNA (as evaluated from cDNA) in the DC differentiated in the presence of NGcGM3, but not in the cells differentiated without the ganglioside. Additionally, the maturation upon an inflammatory stimulus with lipopolysaccharide (LPS) of DC differentiated in the presence of NGcGM3 was impaired, as shown by a 50% reduction of the expression levels of the co-stimulatory molecule CD40 [13].

In order to study the direct influence of NGcGM3 on DC maturation, DC cells differentiated from their bone marrow precursors were stimulated with LPS in...
the presence of the ganglioside. NGcGM3 was also able to directly affect DC maturation, evidenced by a reduction in the levels of the co-stimulatory molecules CD40, CD80 and CD86. Furthermore, the production of IL12 by these cells decreased four-fold when stimulated with LPS in the presence of the ganglioside (Figure 2d). IL12 is a key player during the induction of a proinflammatory profile in Th lymphocytes by DC.

The effect of NGcGM3 on DC differentiation or maturation as reflected on their capacity for modulating the production of cytokines in Th lymphocytes was also studied. CD4+CD25+ Th cells were co-cultivated with DC differentiated or matured in the presence of the ganglioside. A similar phenomenon was detected when the frequency of IL10-producing CD4+ T-lymphocytes was measured instead. The presence of NGcGM3
during stimulation lead, at a minimum, to a three-fold increase in the percentage of cells expressing this cytokine, and again, previous differentiation of the DC in the presence of the ganglioside further augmented the frequency of IL10-producing T lymphocytes by 20% [13]. The results suggest that although the presence of NGcGM3 is absolutely necessary for a detectable change in the response of Th lymphocytes upon a stimulus such as TCR-mediated signaling, its influence on DC also extends to the promotion of an anti-inflammatory cytokine secretion profile. This finding underscores the potential significance of metastasis to secondary lymphoid organs within the context of tumor interaction with the immune system, where the buildup of ganglioside shed from tumor cells would have a direct influence on the activation of lymphocyte populations.

**Conclusions**

Considering the importance of DC and Th lymphocytes as coordinators of the specific immune response, it can be asserted that NGcGM3 has a negative influence on the cellular populations that guarantee the efficacy of immune surveillance on cancer. Our results as a whole validate the N-glycolylated variant of GM3 as a target for cancer immunotherapy; based not only on its preferential expression in human tumoral cells, but also on its relevance for tumor biology. There are antitumoral diagnostic and therapeutic strategies already under development based on this target.