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Polycationic carbosilane dendrimer decreases angiogenesis and tumor-associated macrophages in tumor-bearing mice.

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ABSTRACT

Therapies against cancer have been improved and progressed during last decades, initially were using chemotherapeutic drugs that directly affect tumor cells, and nowadays are focused in cellular therapies aimed to treat the tumor stroma; since tumor and stromal cells are jointly controlling development and tumor progression. Immunotherapy achieves a great relevance since could modify tumor stroma controlling tumor growth. Tumor-associated macrophages have been proposed as target cells owing to the positive correlation between high content of macrophages and the bad prognosis to tumor development. The 2G-03NN24 dendrimer had previously shown immunomodulatory effects reducing the functional capabilities of human anti-inflammatory macrophages, leading them to a pro-inflammatory state, and thereby helping to control tumor development. New dendrimer skills against tumor mass are described in presented *in vivo* studies using tumor-bearing mice. MC38 cells were used to induce tumors in C57BL/6 mice. Tumor growth was evaluating during 21 days and tumors were stained with hematoxylin/eosin to analyze the histopathology features. Tumor histopathology studies show that 2G-03NN24 dendrimer decreases the tumor size and the number of intratumoral blood vessels. Further, cellular populations on tumor mass were analyzed by an immunofluorescence assay. Evaluation of tumor-associated macrophages indicates that 2G-03NN24 dendrimer reduces the amount of tumor-associated macrophages, creating a more favorable microenvironment within tumors. Data defines the 2G-03NN24 as a candidate for finding a new antitumor compound based on cellular therapies.

KEYWORDS: Carbosilane dendrimer, 2G-03NN24, tumor-associated macrophages, antitumor compound, cellular therapy, immunotherapy.

INTRODUCTION

Tumors are formed by cancer-initiating cells and other type of cells which are derived from this initiating group.[1] In addition to tumor cells, tumor also contains a stroma, composed by fibroblasts, endothelial cells, mononuclear cells, tumor-associated macrophages (TAM), lymphocytes, activated B cells and neutrophils. Tumor and stromal cells are jointly involved in tissue destruction and repair, controlling development and tumor progression.[2] These cells induce the cell recruitment into the tumor, providing an excellent environment to improve its survival. [3,4]

Conventionally, chemotherapeutic drugs are the habitual treatment in the clinical practice. However, recent breakthroughs in the struggle against tumors are aimed towards the cellular therapy as a new alternative and complementary cancer immunotherapy.[5,6] TAM have been proposed as target cells for the treatment of tumors owing to the great relevance that they have in a tumor context.[7] The contribution of macrophages to tumor development is inferred from the poor outcome associated with enhanced levels of M-CSF (macrophage colony stimulating factor) and progression to metastasis seen in $Csf1^{op/op}$ mice, and the positive correlation between high content of TAM and the bad prognosis.[8-10] Macrophages present modifiable phenotypes, they are capable of destroying cancer cells and promoting antitumor immune responses, but can also contribute to tumor progression by enhancing tumor cell survival, migration, invasion and dissemination.[11] Analysis of the effector functions of TAM indicates that, in most cases, macrophages contribute to tumor initiation secondary to chronic inflammation by virtue of their proinflammatory M1-related functions, whereas they support tumor progression through trophic and immunosuppressive M2-related activities.[12-14] So, at the early stages of inflammation-induced tumors, macrophage-derived proinflammatory cytokines are needed for tumor initiation and growth. Then, as

tumor progresses, and under the influence of tumor-derived factors (M-CSF, VEGF, IL-10), TAM develop an "alternative"/M2 immunosuppressive profile, with TAM-derived factors contributing to suppression of tumor-specific immune responses.[15,16] However, TAM in regressing tumors exhibit proinflammatory tumoricidal properties.[17,18] All of above, one of the main objectives in immunotherapy against tumors is to decrease anti-inflammatory functions and skewing the macrophages phenotype to pro-inflammatory cells.[19]

Following in this field, a recent report published by our group described the development of a new nanoparticle called 2G-03NN24 dendrimer, and its ability to modify the anti-inflammatory cells inducing a phenotype-switch in human macrophages.[20] Having determined the phenotypic and functional changes that the 2G-03NN24 carbosilane dendrimer produced on human macrophages that were used as a model of TAM, we have performed *in vivo* experiments to study whether this dendrimer could modify the tumor in an *in vivo* murine model.

In this study we present results obtained when tumor-bearing mice were treated with the 2G-03NN24 dendrimer, suggesting that it could be used as nanomaterial having a therapeutic effect in an *in vivo* model.

EXPERIMENTAL SECTION

Dendrimer

The third generation cationic carbosilane dendrimer used was [G2O3(SiONN)12]24+, referred as 2G-03NN24 (figure 1). Synthesis has been previously reported.[21]

Induction of tumors and dendrimer treatment in C57BL/6 mice

MC38 cells were cultured with 10% FBS supplemented RPMI medium. Cell culture was harvested, centrifuged and resuspended in PBS. C57BL/6 mice (5 mice at 8 weeks old) were used to inject subcutaneously into the back $5x10^5$ MC38 cells (100µL) per mouse. After three days, 5 mice were treated with a peritumoral injection of 2G-03NN24 dendrimer (0,5mg/kg) or with 100µL PBS (phosphate-buffered saline). Treatment was repeated every 24 hours for 18 days. During this time tumor growth was evaluated by measuring the size of tumor mass every 24 hours. On day 21 post-injection of MC38 cells, mice were sacrificed. All protocols were approved by the Centro de Investigaciones Biológicas/Consejo Superior de Investigaciones Científicas Ethics Committee.

Histopathology studies

Tumors of control and treated mice were removed, washed once with PBS, included in 4.0% formaldehyde buffered to pH=7 and stabilized with methanol. Sections of the tumor mass were embedded in paraffin wax, using alcohols with increasing degree, two baths of xylene and one bath of paraffin wax inside a cast. Subsequently they were cut using a microtome. For dewaxing, tumors were immersed in 2 baths of xylene (10 minutes) and 3 in descending order of alcohols (100%, 90% and 70%) for 5 minutes. Then, tumors were stained with hematoxylin (5 minutes) and eosin (5 minutes). For dehydration process, were used again alcohols (70%, 96% and 100%) and xylene solution. Tumors were mounted with DPX mounting media. To calculate the parameters of mitotic index, peritumoral macrophages and intratumoral blood vessels, 10 fields (160 µm x 119 µm per field; 40X high power field) of each sample were evaluated.

Immunofluorescence

Tumors were removed, washed once with PBS and embedded in Tissue-Tek O.C.T. (optimum cutting temperature) compound for freezing with liquid nitrogen. Subsequently cryosections for immunofluorescence studies were assayed. Slides with the included samples were thawed, rehydrated with PBS and blocked with RPMI 1640, 10% FBS (fetal bovine serum), 1% BSA (bovine serum albumin), 100 μg/ml human IgG (immunoglobulin G) and Triton X-100 for one hour. Slides were incubated with 1:100 rabbit anti-inducible nitric oxide synthase (iNOS) antibody for one hour, and after with 1:1000 Cy(cyanine)3-labeled anti-rabbit antibody. To detect macrophages, 1:100 anti-F4/80-Cy5 antibody and DAPI (4',6-diamidino-2-phenylindole) were used. Slides were prepared with mounting medium (Dako), and stored for subsequent analysis by confocal microscopy.

Statistical analysis

Results were analyzed with the nonparametric test U Mann-Whitney, to take into account the variability between conditions that may be due to intrinsic differences presented among the mice. Results are shown as average or median and 10-90 percentiles according to the experiments. Differences that were considered statistically significant were indicated as *$p<0.05$.

RESULTS AND DISCUSSION

The relevance of the immune system against tumors is well reflected showing the numerous inhibitory networks that tumor mass present to reduce antitumor immunity.[5] Immunotherapy against cancer is aimed to attack the tumor through the stimulation of the immune system.[5,22-24] The 2G-03NN24 dendrimer (figure 1) had shown immunomodulatory effects reducing the functional capabilities of human anti-inflammatory macrophages, leading them to a pro-inflammatory state.[20] To study

whether the 2G-03NN24 dendrimer was modifying the TAM in an *in vivo* model and therefore could be affecting the tumor development, subcutaneous solid tumors were induced in C57BL/6 mice and treated with 2G-03NN24. Tumor growth was evaluated, showing that PBS-treated C57BL/6 mice present tumors up to 420 mm^3 (mean of 195 mm^3), while tumor size in 2G-03NN24-treated mice were reaching up to 320 mm^3 (mean of 150 mm^3) (figure 2A and 2B). Therefore, 2G-02NN24 dendrimer decreases the tumor size and further the amount of TAM (figure 3B), processes strongly related, since TAM depletion suppresses tumor growth.[25]

One of the greatest challenges today is to maintain tumor cells confined inside tumor mass and therefore prevent metastasis. To achieve it, the most relevant process to be controlled is the angiogenesis, which is required to nourish and oxygenate all parts of the tumor, but unfortunately allows tumor cells go out of the tumor and facilitates cellular infiltration inside the tumor.[24,26] Tumor histopathology studies show that 2G-03NN24-treated mice were decreasing intratumoral blood vessels (IBV) (figure 3A), indicating a reduction in angiogenesis process, and further present a fewer number of TAM (figure 3B), reflecting the low cell infiltration due to the reduction of intratumoral blood vessels. These results are in agreement with authors that relate the higher density of TAM with more aggressive tumors, greater vascularization and metastatic phases, due to the release of cytokines, chemokines and proteases by TAM that enhance angiogenesis.[24,26-32] Our results indicate a potential effect by dendrimer on decreasing angiogenesis process and indeed metastasis. On the other side, the 2G-03NN24 dendrimer treatment was not affecting at number of mitotic figures (figure 3C), indicating that was not over-induced tumor cell proliferation and therefore neither induced tumor growth (figure2).

On the other side, TAM contribute to tumor initiation secondary to chronic inflammation by virtue of their proinflammatory M1-related functions, whereas they support tumor progression through trophic and immunosuppressive M2-related activities.[12,13] Further, TAM in regressing tumors exhibit proinflammatory tumoricidal properties.[17,18] All of above, one of the main objectives in immunotherapy against tumors is skewing the macrophages phenotype to pro-inflammatory cells.[19] In order to evaluate the effect of the 2G-03NN24 dendrimer over macrophages phenotype, an immunofluorescence assay was performed. Tumor mass photographs show that 2G-03NN24-treated mice present a higher inducible nitric oxide synthase (iNOS) expression than mice treated with PBS as controls (figure 4). Further, increased expression of iNOS partially colocalized with F4/80+ cells indicates that the 2G-03NN24 dendrimer was promoting the expression of iNOS by TAM (F4/80+iNOS+, yellow, figure 4). Our data is in agreement with authors whose described that iNOS protein is typically expressed by proinflamatory macrophages that present antitumor properties.[19] On the other side, it was observed cells with F4/80-iNOS+ phenotype indicating a rising of iNOS by other cell populations in addition to TAM, such as stromal or tumor cells (figure 4). Other authors suggest that iNOS activity could promotes angiogenesis, macrophage infiltration and tumor growth, but our results do not show these relations, reason why this is a controversial point.[33,34] The increase of iNOS protein could be a consequence of the hypoxic condition created by the reduction on blood vessels number, although it cannot be excluded a direct effect by dendrimer over cells.[35]

CONCLUSIONS

Our findings make the 2G-03NN24 treatment very encouraging, because suggest a role for dendrimer modulating the tumor microenvironment and decreasing the probability

of metastasis. Data defines the 2G-03NN24 as a candidate for finding a new antitumor compound based on cellular therapies.

ABBREVIATIONS

BSA: bovine serum albumin

Cy: cyanine

DAPI: 4',6-diamidino-2-phenylindole

FBS: fetal bovine serum

IBV: intratumoral blood vessels

IgG: immunoglobulin G

IL: interleukin

iNOS: inducible nitric oxide synthase

M-CSF: Macrophage Colony Stimulating Factor

PBS: phosphate-buffered saline

TAM: tumor-associated macrophages

TGF-β: Transforming Growth Factor Beta

VEGF: vascular endothelial growth factor

ACKNOWLEDGMENTS

The authors thank R. Samaniego for expert technical assistance (unit of Confocal Microscopy, HGUGM). This work has been (partially) funded by the RD12/0017/0037, project as part of Acción Estratégica en Salud, Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica 2008-2011 and cofinanced by Instituto de Salud Carlos III (Subdirección General de Evaluación) and Fondo Europeo de Desarrollo Regional (FEDER). RETIC PT13/0010/0028, Fondo de Investigacion Sanitaria (FIS) (grant number PI13/02016), CTQ2011-23245 and CTQ2014-54004-P

(MIMECO), Comunidad de Madrid (grant numbers S-2010/BMD-2351 and S-2010/BMD-2332], CYTED 214RT0482. CIBER-BBN is an initiative funded by the VI National R&D&i Plan 2008-2011, IniciativaIngenio 2010, the Consolider Program, and CIBER Actions and financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund. This work was supported partially by a Marie Curie International Research Staff Exchange Scheme Fellowship within the 7th European Community Framework Programme, project No. PIRSES-GA-2012-316730 NANOGENE, co-financed by the Polish Ministry of Science and Higher Education (grant No. W21/7PR/ 2013).

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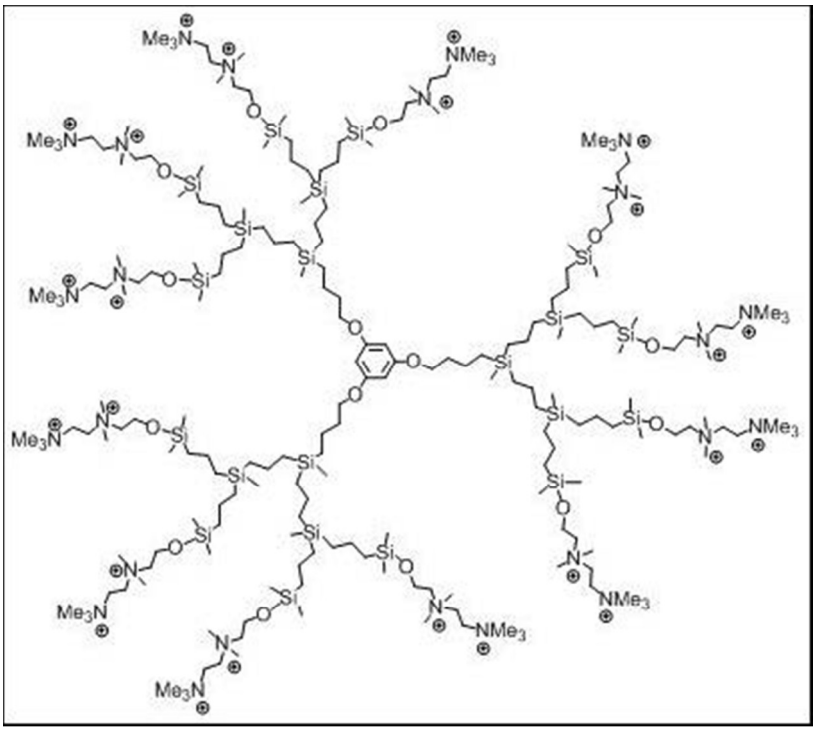
FIGURE LEGENDS

Figure 1.- Schematic representation of dendrimer structure. 2G-03NN24 carbosilane dendrimer is shown.

Figure 2. Tumor growth in C57BL/6 mice. A) Individual values of daily measures on C57BL/6 mice treated with PBS as controls (•) and treated with 2G-03NN24 dendrimer (•) are shown. The daily mean value of control mice (n=5; –•–) and 2G-03NN24 mice (n=5; –•–) are indicated. B) Tumor size at the end point of the experiment. Low-high bar graphs with mean are shown. Mann-Whitney test #p=0.05476.

Figure 3. Histopathology studies of tumors. Photographs of control C57BL/6 mice (PBS) and 2G-03NN24-treated mice are shown; box and whiskers graphs represent the histopathology parameters using median and 10-90 percentiles. (A) Photographs: intratumoral blood vessels are shown (40X); Right side: number of intratumoral blood vessels (IBV) per field (160 μm x 119 μm) are indicated. (B) Photographs: TAM are shown (40X); Right side: number of peritumoral TAM per field (160 μm x 119 μm) is indicated. (C) Photographs: cells where mitotic figures could be observed are shown (50X); Right side: mitotic figures per field (160 μm x 119 μm) are represented. Mann-Whitney test *p<0.05.

Figure 4. TAM phenotype. Immunofluorescences of one representative PBS or 2G-03NN24-treated C57BL/6 mouse are shown. DAPI labeling cell nuclei (blue), TAM (F4/80+; red) and proinflammatory macrophages (iNOS+; green) are shown. Merge images were made by overlapping F4/80 and iNOS labeling images.



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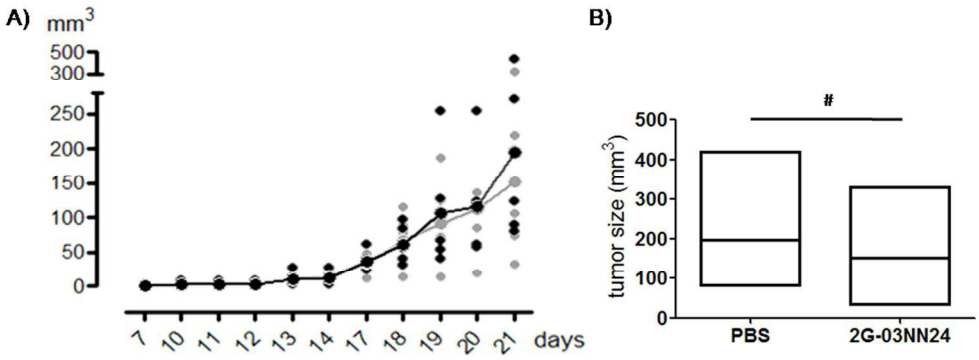

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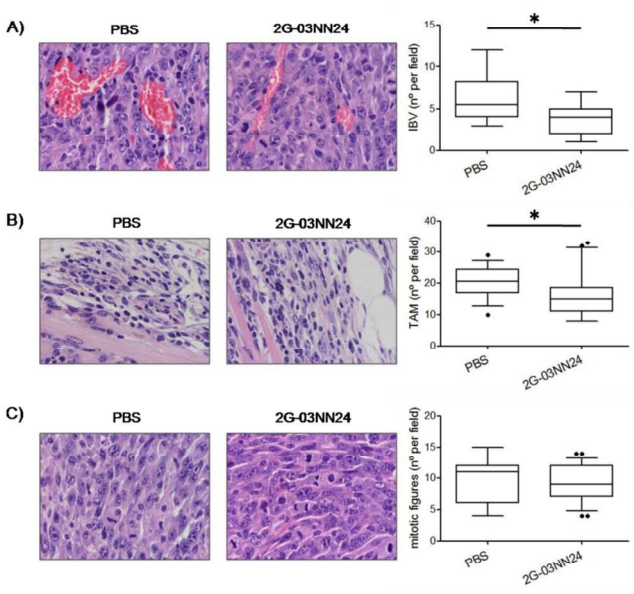

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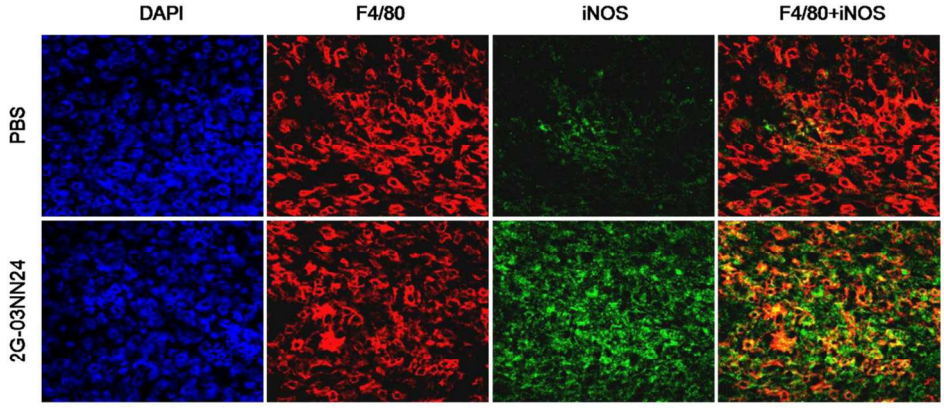

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