IMPLANT MICROPARTICLES - A NEW CONCEPT FOR NON-INVASIVE CANCER THERAPY

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ABSTRACT

Some progress in cancer research was possible in recent years mainly due to important advances in nanotechnology. However, clinical use of nanomaterials is still hindered by limitations. In search of better performance and control of inoculated materials, the efficiency and toxicity of SBBC implant particles was assessed. B16 tumoral cells (murine melanoma) were subjected to SBCC particles using *in vitro* and *in vivo* experimental models. *In vitro* experiments concerning the inhibition growth of tumoral cells using SBCC particles were performed by Flow Cytometry and by MTT Assay. *In vivo* experimental model (C57BL/6 mice) was used to complete this investigation: weight, viability and tumoral dimension were monitored. An anti-proliferative activity on B16 tumoral cells and an ability to produce apoptosis were observed. A reduction of tumoral volume and a 54% survival rate in the treated animals compared to the controls was obtained. Our preliminary results showed that the SBCC implants were effective against B16 melanoma cells, while there is no toxicity associated.

Key words: tumor, anti-proliferative, Flow Cytometry, SBCC implant particles

INTRODUCTION

Chemotherapy is associated with local and systemic side-effects. Some progress in cancer research was possible in recent years mainly due to important advances in nanotechnology. From biofunctionalized quantum dots or nanotubes [1], nanoantennas [2] and nanoshells to magnetic nanoheaters and nanoparticle carriers [3, 4], the fast development of nanomaterials has lead to important achievements in the fight against cancer [5,6].

The development of innovative treatments using such materials may involve stricter regulations for clinical research than the current ones [7].

Despite of the high spending in cancer nanotechnology research, there are still many issues and challenges to be addressed before using nanomaterials in clinical practice [8]. The size of the nanoparticles has great benefits in the interference with intracellular activity, but their size induces limitations like: poor selective toxicity, poor penetration control within the tumor and also poor localization control within the body. Moreover, their aggregation in vital organs leads to organ toxicity and death [6]. We imagined that ideal antitumoral agents would be effective nontoxic microparticles of sizes sufficiently large so that no free circulation within the body is possible. Knowing that normal vasculature is 8-10 μm in diameter and tumor vasculature ranges from 20 to 100 μm, we have used SBCC implant particles of 400 μm in size for investigations on antitumoral effect [9].

MATERIALS AND METHODS

SBCC implant particles

Proprietary implant particles with sizes in the submillimetric range. Their preparation method is patent protected [6].

Cell Lines

Murine cell line (B16-melanoma) was grown under standard conditions in an incubator with 5% CO_2, in Dulbecco's Modified Medium (DMEM), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units per ml penicillin and 100 μg/ml streptomycin.

MTT Viability / Cytotoxicity Assay

$5x10^4$ B16 cells/100 μl/well were cultured in flat-bottomed 96 well tissue culture plates for 4 hours at 37°C and 5% CO_2, then treated with SBCC implant

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microparticles. After 72 hours, the plate was centrifuged at 800 g for 5 minutes and the supernatants taken out. 50 ml/well MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), 1 mg/ml in phosphate buffer saline (PBS) solution, were added to each well. MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals [10]. After 4 hrs incubation at 37°C and 5 % CO_2, the plates were centrifuged 5 min. at 800 g and the supernatants were removed. The reaction was stopped with 50 ml/well Sodium Dodecyl Sulfate solution. The plates were kept overnight at 37°C and then the absorbance was measured at 540 nm, using a THERMO Multiskan spectrophotometer. Average values for triplicate samples were calculated.

Flow Cytometry

Tumoral B16 adherent cells were cultured in Dulbecco's Modified Medium (DMEM) to obtain a monolayer and then released by trypsinization. After centrifugation at 2000 rpm for 2 minutes at 2-8°C, pelleted cells were resuspended at the working concentration ($1x10^6$ cells /sample). In order to study the apoptosis process, B16 tumoral cells were treated with SBCC implant particles, then incubated for 24 h at 37°C and 5% CO_2 and then the cells were washed in PBS buffer. Cells were gently resuspended in 1.5 ml hypotonic fluorochrome solution (Propidium Iodide, 50 µgml in 0.1% sodium citrate plus 0.1% Triton X-100) in 12x75 polypropylene tubes (Becton Dickinson). The tubes were placed at 4°C in the dark overnight, before flow-cytometric analysis (FACScalibur Becton Dickinson system). The data were analyzed with WinMDI software.

***In vivo* Experiments**

Six groups of C57BL/6 mice, female, weighing 18-20.1 g, housed in the Baneasa Animal Facility SFP area for rats/mice of Cantacuzino National Institute of Research and Development for Microbiology and Immunology, were used according to the protocol presented in Table 1. Day 11 was considered the day of tumor appearance.

Each mouse in Group 3 received 5 implant particles subcutaneously, simultaneously with the tumoral cells. All 5 particles were inoculated in the same place. The weight, viability and tumoral dimension were monitored every fourth day after inoculation, for 25 days. The last day of our experiment was the day when all animals in Group 2 (positive control) were dead (Day 23).

Finally, the mice were euthanized and dissected. Anatomo-pathological examination was also performed in all animals, both on the dead ones and at the end of the experiment.

The tumor volume was quantified by measuring its length (L) and width (l) with a vernier caliper, using the following formula: $V = (L \times l^2)/2$. The unit measure is cm^3.

The animals were kept under standard conditions, temperature 18-24°C, UR 45 - 65%, cycle of lighting 12/12. The food and the water were administered *ad libitum*.

The *in vivo* experiments were undertaken in accordance with the national and international regulations concerning animal testing. The protocol was approved by the ethics committee of Cantacuzino National Institute of Research and Development for Microbiology and Immunology.

RESULTS

Our results were focused on the effects of SBCC implant particles either on the viability of tumoral cells or on live animals.

Table 1. Inoculation protocol used in the *in vivo* experiment.
Implant particles (IP), Tumoral cells (TC)

Group 1-control			Group 2-positive control			Group 3		
No. of mice	Day 1	Day 11	No. of mice	Day 1	Day 11	No. of mice	Day 1	Day 11
5	-	-	13	$2.5x10^5$ TC cells/100µl/mouse	-	13	TC + IP simultaneously	-

Group 4			Group 5			Group 6		
No. of mice	Day 1	Day 11	No. of mice	Day 1	Day 11	No. of mice	Day 1	Day 11
15	TC	IP - peritumoral	14	TC	IP - intratumoral	5	-	IP

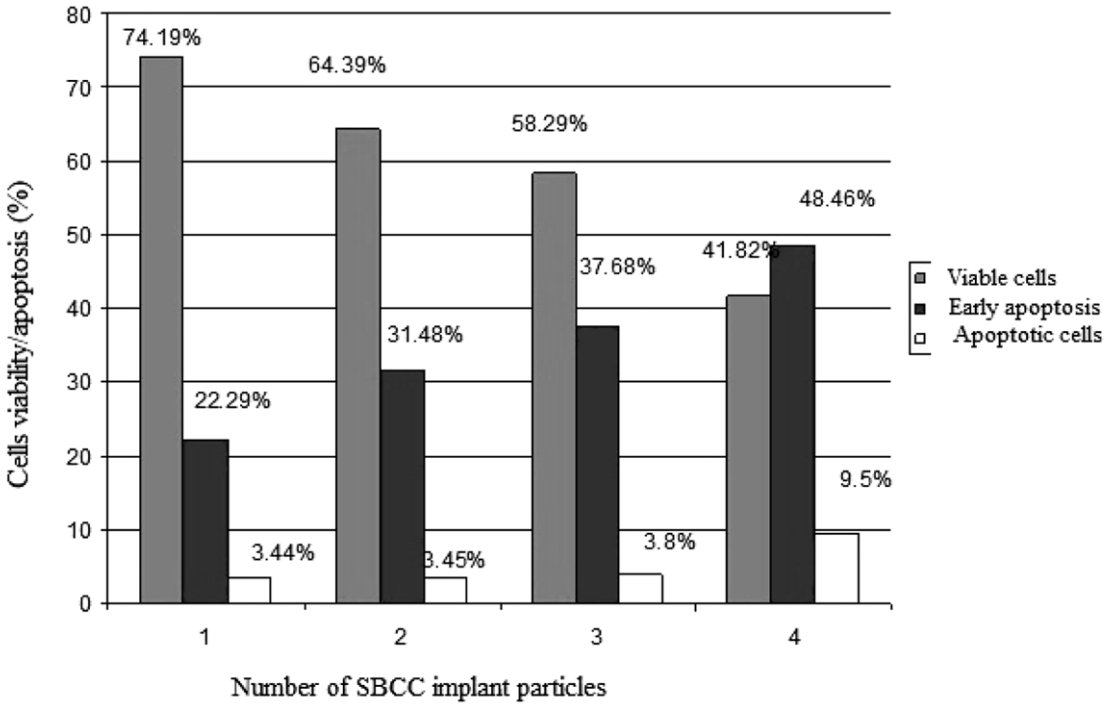

Fig. 1. Experimental results obtained by Flow Cytometry

Flow Cytometry analysis

Flow cytometry analysis showed that SBCC implants had apoptotic effects proportional to the number of particles used (see Fig. 1).

As it could be seen in Fig. 1, the percent of apoptotic cells was dependent of the number of SBCC particles, with values between 3.44% and 9.5%. Early apoptosis had a greater significance, but it should be taken into account the fact that the SBCC particles treatment was applied for 24 hours. This result points out to also investigate a longer time contact between SBCC particles and tumoral cells.

Effect of SBCC implant particles on B16 cells viability

The growth/inhibition of tumor cells by SBCC implants had a polynomial increase with the number of particles used, as observed in Fig. 2. Experiments were assessed by MTT assay.

We could mention that SBCC particles had no toxic effect on B16 tumor cells.

Effect of SBCC implant particles in mice

The *in vivo* experimental model has revealed that no lessions or infections were formed in any of the animals used.

The presence of implant particles in healthy tissue resulted in the appearance of a granuloma and all inoculated animals were viable. Fig. 3 presents an image of the granuloma formed around the SBCC implants, twenty one days after inoculation (mice Group 6).

Dissection of the granuloma revealed the microparticles and the surrounding fatty tissue.

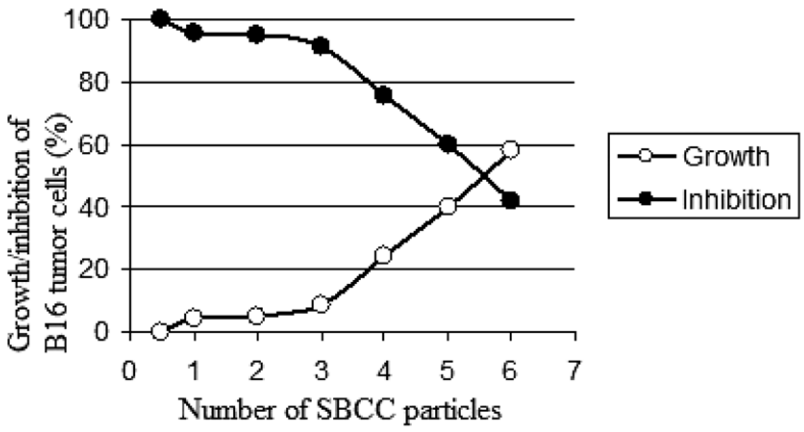

Fig. 2. Effect of SBCC particles on B16 tumor cells viability

Fig. 3. Granuloma formed in healthy tissue (mice Group 6) due to the presence of SBCC particles, three weeks after inoculation

The first tumors appeared on the 11th day after inoculation (Fig. 4). It is a striking result the fact that visible tumors appeared only in 22% of the mice of Group 3 (those treated with SBCC particles from the first moment of inoculation of the tumoral cells), whereas 86% of the mice in the untreated Group 2 presented tumors on the 11th day.

The mean value of tumor volume on each day of measurement was calculated for each animal group and plotted against time, starting with Day 11 (Fig. 5). As can be observed, the mean value of tumor volume was lower in all treated animals compared to the positive control Group 2. The percentage of dead animals on the last day of measurement (the 23rd day) in groups 3, 4 and 5 was 44.3%, 68.4% and 71.7% for the positive control Group 2 (100%), as can be also observed in Fig. 5. These results showed an antitumoral effect of the treatment.

Fig. 7 gives the percentage of dead animals in each group, on each day of measurement. The mortality rate was lower in all treated groups compared to Group 2. It was a nice surprise to see that the simultaneous inoculation of the tumor cells and the SBCC implant particles (Group 3) inhibited tumor growth to such an extent, that 45% of the animals were still alive when all the untreated ones were dead, e.g.

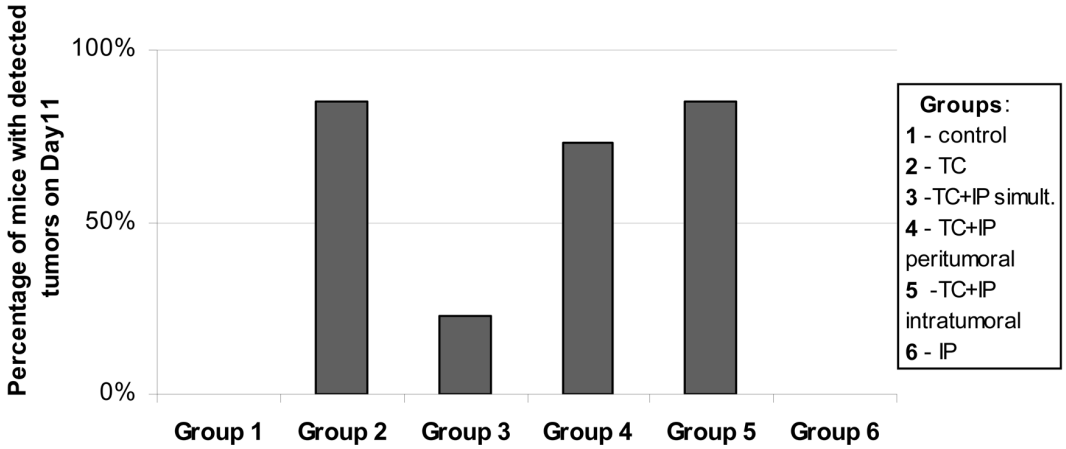

Fig. 4. Tumors appearance on Day 11. TC - tumoral cells, IP - implant particles

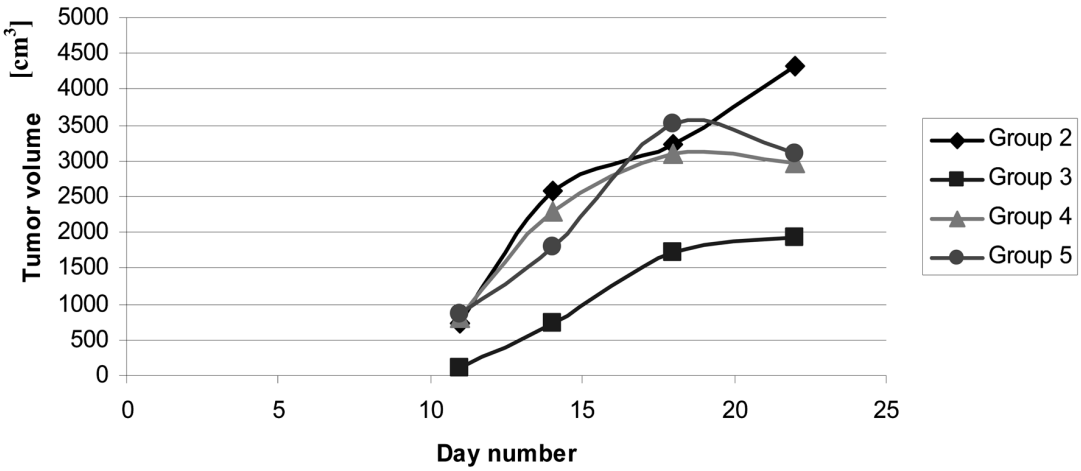

Fig. 5. Time evolution of tumor volume for each animal Group

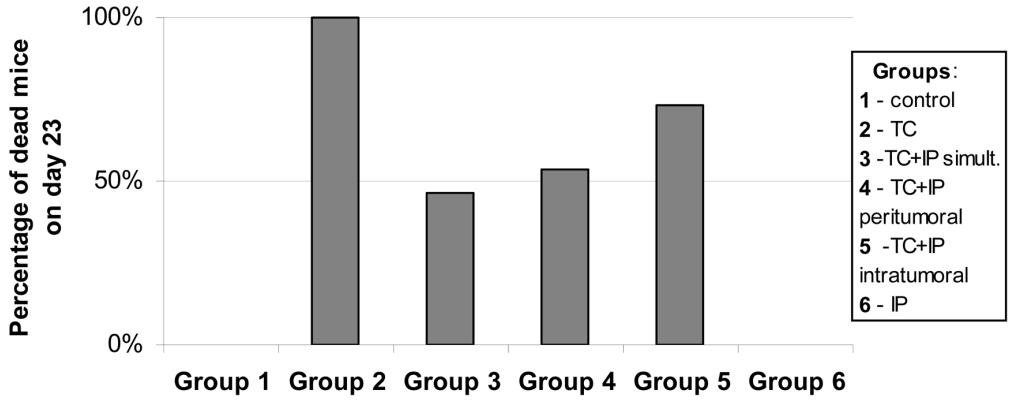

Fig. 6. Percentage of dead animals on Day 23 for all animal Groups

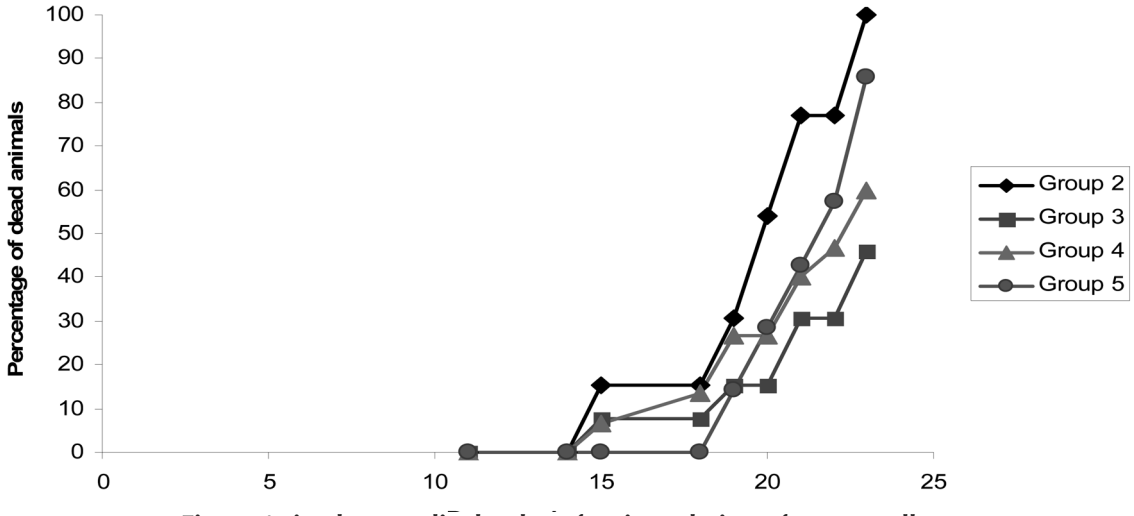

Fig. 7. Animals mortality by day after inoculation of tumor cells

on Day 23. In 31% of the survivors, the tumor volume decreased down to immeasurable values.

The weight gain within the screening period (15 days) of the reference Group 1 was between 5 and 9 g. In this period of time, the tumors typically grew up to 4.5 - 5 g. The weight of the animals in Groups 3, 4 and 5 reflected the increase of tumor, e.g. 10 - 14 g.

DISCUSSION

Although the etiology of different cancers varies, all are associated with uncontrolled cellular growth. Scientists are in a continuous search for designed materials that are able to efficiently but safely destroy tumor cells. We demonstrated here the efficiency of SBCC implants to reduce tumor growth while safely remaining at the inoculation site.

Our *in vivo* and *in vitro* results have shown an inhibitory effect of SBCC implants on B16 tumor cells. The size of the SBCC implant particles did not allow free circulation within the body, their agglomeration in vital organs being thus avoided, in contrast with nanomaterials. Another benefit of using SBCC implants to inhibit tumor growth was that they could be removed via surgical intervention once their role was accomplished.

A low local toxicity of the healthy cells is ensured by the immune system which walls-off the SBCC implants by forming a granuloma of fatty tissue arround them, as evidenced in our *in vivo* experiment. Also, the weight gain and viability of the animals inoculated with SBCC implants was identical to the reference group.

It was also important to note that no macroscopic modifications of any organ or tissue were observed as a result of the presence of the SBCC implants (data not shown).

It is estimated that the hospitalization time can be as low as a few days, once the treatment protocol is optimized. We have strong reasons to believe that the use of such particles in the treatment of solid tumors is a promising very effective and least invasive therapy for cancer. Further studies remain to prove that.

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