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РОЛЬ ГАЛЕКТИНА-3 В МЕТАСТАЗИСЕ ОПУХОЛЕЙ

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Мы использовали метастатический вариант клеток B16 меланомы (B16F1) чтобы изучить развитие метастаз в легких у галектин-3-дефицитных (gal-3$^{-/-}$)-мышей на почве C57BL/6. Исследования в условиях *in vivo* показали, что по сравнению с (gal-3$^{+/+}$)- мышами (gal-3$^{-/-}$)-мыши проявляли устойчивость к колонизации легких клетками меланомы B16F1 (p<0.03). Исследования в условиях *in vitro* показали более высокое число прикрепленных злокачественных клеток, связанных с эндотелием легких у (gal-3$^{+/+}$)-мышей (p<0.001) и более высокую опухолево-специфическую цитотоксичность спленоцитов у (gal-3$^{-/-}$)-мышей, которым была привита опухоль. Это показывает, что манипуляция галектином-3 может представлять собой новый терапевтический подход в лечении злокачественных заболеваний.

Ключевые слова: *галектин-3, злокачественная меланома, метастазы, B16F1.*

Galectin-3, a lectin with specificity for beta galactosides, that is overexpressed in a variety of tumour and immune cells in response to various stimuli. This protein interacts with a numerous complementary glycoconjugates and regulates many biological functions and signaling pathways in normal and cancer cells. The expression of Galectin-3 is modulated in many different tumour types [1] and, in general, expression of this protein is associated with poor prognosis and acquisition of a metastatic phenotype [2]. For example, its overexpression in human melanoma correlates with metastatic progression and with negative clinical outcome [3]. Recent observations from Krishnan et al. [4] suggest that Galectin-3 constitutively expressed on the lung vascular endothelial cells plays a key role in the adhesion of circulating murine melanoma cells to lungs. During the last years, an extensive accumulation of data has changed the perspective of this multifunctional protein. It was therefore postulated that Galectin-3 is involved in tumour progression and metastasis by modulating various biological events, including cell adhesion, migration, angiogenesis, and immune escape [2, 5].

Galectin-3 have recently attracted the attention of as novel regulator of immune cell homeostasis [6, 7]. Among the 15 galectin members, Galectin-3 is expressed in many immunocompetent and inflammatory cells including macrophages and activated T lymphocytes [8–10]. Galectin-3 affects differentiation and growth of various immune cells: it activates several lymphoid and myeloid cells, such as mast cells, neutrophils, monocytes and T cells, resulting in mediator release, superoxide anion production, and cytokine production [11–13]. Galectin-3 has been shown to induce apoptosis in T lymphocytes, including human T leukaemia cell lines, human peripheral blood mononuclear cells (PBMC), and activated mouse T cells [14,15]. Zubieta MR. et al. [16] demonstrated that Galectin-3 expression correlated with apoptosis of tumour -associated lymphocytes in human melanoma biopsies. A recent study also found that Galectin-3 secreted by tumours facillitates tumour immune escape by killing tumour reactive CD8+T cells and promotes tumour growth in a mouse model of colorectal cancer [17].

However, there is still scarce information available on how Galectin-3 regulates metastasis *in vivo*. The hematogenous phase of metastasis is a dynamic and coordinated multistep process. During this phase, the tumour cells are directly confronted with effector mechanisms of the host immune system [18]. It appears that one of the critical steps in hematogenous phase of metastasis is the adhesion of circulating tumour cells to the vascular endothelium in targeted organs. This process is thought to be regulated by the specific expression of various adhesion molecules and their ligands on the surface of tumour cells and endothelial cells [19]. In this regard, some evidence indicate that Galectin-3 and its glycoconjugate ligands are engaged in this process [5]. We used metastatic variant of B16 melanoma (B16F1) to

study lung colonization and tumor cell adhesion in order to directly demonstrate its relevance for disease progression *in vivo*. In addition, we examined *in vitro* cytotoxic activity of adherent and non -adherent cell populations isolated from spleen of gal-$3^{-/-}$ mice vs. gal-$3^{+/+}$ mice.

Materials and methods

Animals. The experiments were approved by the ethics board of the Medical Faculty of Kragujevac. We used Galectin-3-deficient (gal-$3^{-/-}$) on C57BL/6 background mice (generated as previously detailed by Hsu et al. (20), kindly provided by Dr Daniel Hui through Prof. FY. Liew, Glasgow, UK), and "wild-type" (gal-$3^{+/+}$) C57BL/6 mice. Male and female mice 8-12 weeks old were used in all experiments.

Cell Culture. The murine skin melanoma cell line B16F1 was purchased from the American Type Culture Collection (ATCC, CRL-6323). The cells were routinely cultured in DMEM supplemented with 10% FBS, 2 mmol/l L-glutamine, 1 mmol/l penicillin/streptomycin, 1 mmol/l mixed non-essential amino acids (PAA Laboratories GmbH), in a humidified incubator at 37°C at 5% CO_2. Cell were routinely subcultured as suconfluent monolayers every 3 days and were not kept in culture for more then five passages.

Experimental metastasis assay. For inoculation, B16F1 melanoma cells were harvested upon ~90% confluency using 0.25% trypsin and 0.02% EDTA in PBS (PAA Laboratories GmbH). Cells were washed once in complete medium and twice in DMEM just before inoculation. The number of viable tumour cells was determined by the trypan blue and only those cell suspension with e"95% viable cells were used. Experimental metastasis assay was performed as described previously [21] by the intravenous injection (lateral tail vein) of 5×10^4 cells, in a volume 0.2 ml of medium, into syngeneic gal-$3^{+/+}$ and gal-$3^{-/-}$ mice. Twenty-one days after tumour cell injection, lung tissues were removed from these mice and examined histologically.

Histopathological analysis of metastatic lung. Hematoxylin-eosin staining was performed using 4μm paraffin-embedded tumour-bearing lung sections. To avoid missing micrometastasis, stained sections from at least three different levels were examined for the presence of lung metastasis. The number and size of metastatic colonies was examined with light microscope by an independent observer.

Adhesion assays. Adhesion assays were performed essentially as described in [22]. Prior to adhesion experiments, the frozen sections of lungs were air dried at room temperature for 45 min. The sections were incubated at 4°C with 100мl of 2% BSA in PBS for 2h. Subsequently, they were layered with $5x10^3$ B16F1 suspended in 100мl of PBS and incubated at 37°C in a humidified CO_2 incubator. After incubation, the sections were gently washed with PBS to remove non- adherent cells. The bound cells were fixed in methanol for 15 min at 4°C, stained with Mayer's hematoxylin & eosin. Number of attached cells were determined by counting 100 non-overllaping microscopic fields at an ×100 magnification and average number of attached cells was counted. Each experiments was repeated at least three times.

Cytotoxicity assay. Ten days after the i.v. injection of 5×10^4 B16F1, mice were killed, spleens were harvested and single-cell suspensions were prepared by passaging spleen through steel and nylon mesh. Subsequently, cell suspension was incubated for 2 hours in complete media on plastic Petri dishes, previously covered with FBS. The non- adherent cells were rinsed off by vigorously washing with DMEM and the adherent cells were collected by gentle scraping with rubber policemen. Isolated adherent and non-adherent cells were used as effector cells (E), and B16F1 melanoma cells were used as target cells (T). Effector cells were plated at varying ratios (T:E: 1:2 and 1:4) in 96-well flat bottom plates, preincubated for 24h with 1×10^4 target cells/well. MTT cytotoxicity assays were performed as previously described (23). The percentage of cytotoxity was calculated as: cytotoxity (%) = [1 – (experimental group (OD)/control group (OD))]× 100.

Statistical analysis. The data were analyzed using SPSS version 13 statistical package. The normally distribution was compared using the Student's *t* test and the no normally distribution was compared using the Mann–Whitney test. Relationships between parameters were examined using calculation of the Pearson's correlation. $p<0.05$ was considered to be statistically significant.

Results

Gal-$3^{-/-}$ mice exhibited significant resistance to lung colonization of B16F1 melanoma cells. Gal-$3^{-/-}$ mice exhibited significant resistance to lung colonization of melanoma cells as evaluated by number of metastasis and cellularity of lung metastatic colonies. In fact, the

average number of lung metastasis counted was 5.83 in the gal-$3^{+/+}$ mice (mean±SEM; 5.83±1.89) and 1 in the gal-$3^{-/-}$ mice (1.00±1.00), respectively (p=0.003; Fig. 1A). We next examined number of malignant cells in the colonies, and when metastatic colonies were categorized into groups based number metastatic cells, we also found statistically significant differences between gal-$3^{+/+}$ and gal-$3^{-/-}$ mice (p=0.026; Fig. 1B).

At day 21 after inoculation all of gal-$3^{+/+}$ mice (6/6, 100%) had numerous lung metastatic colonies, while only 1 of 5 gal-$3^{-/-}$ mice developed lung metastatic colonies. Beside the difference in number of detected metastatic colonies in lung, we also noticed great difference in size of those colonies (Fig. 1C).

In vitro adhesion of malignant cells is decreased in lung tissue of ***gal-$3^{-/-}$ mice.*** We examined *in vitro* role of the Galectin-3 in the adhesion of the malignant cells onto lung tissue by adhesion assay. Adhesion assay were performed on the frozen lung sections of healthy gal-$3^{+/+}$ and gal-$3^{-/-}$ mice. Consistent with *in vivo* study, our results showed lower capacity of malignant cells to bind onto lung tissue of gal-$3^{-/-}$ mice may contribute to this resistance of mice to melanoma metastasis. The results are shown in Figure 2).

Lymphoid cells derived from Galectin-3- deficient mice show enhanced cytotoxicity toward melanoma cells. In order to investigate the involvement of anti - tumour immune response in malignant melanoma, we

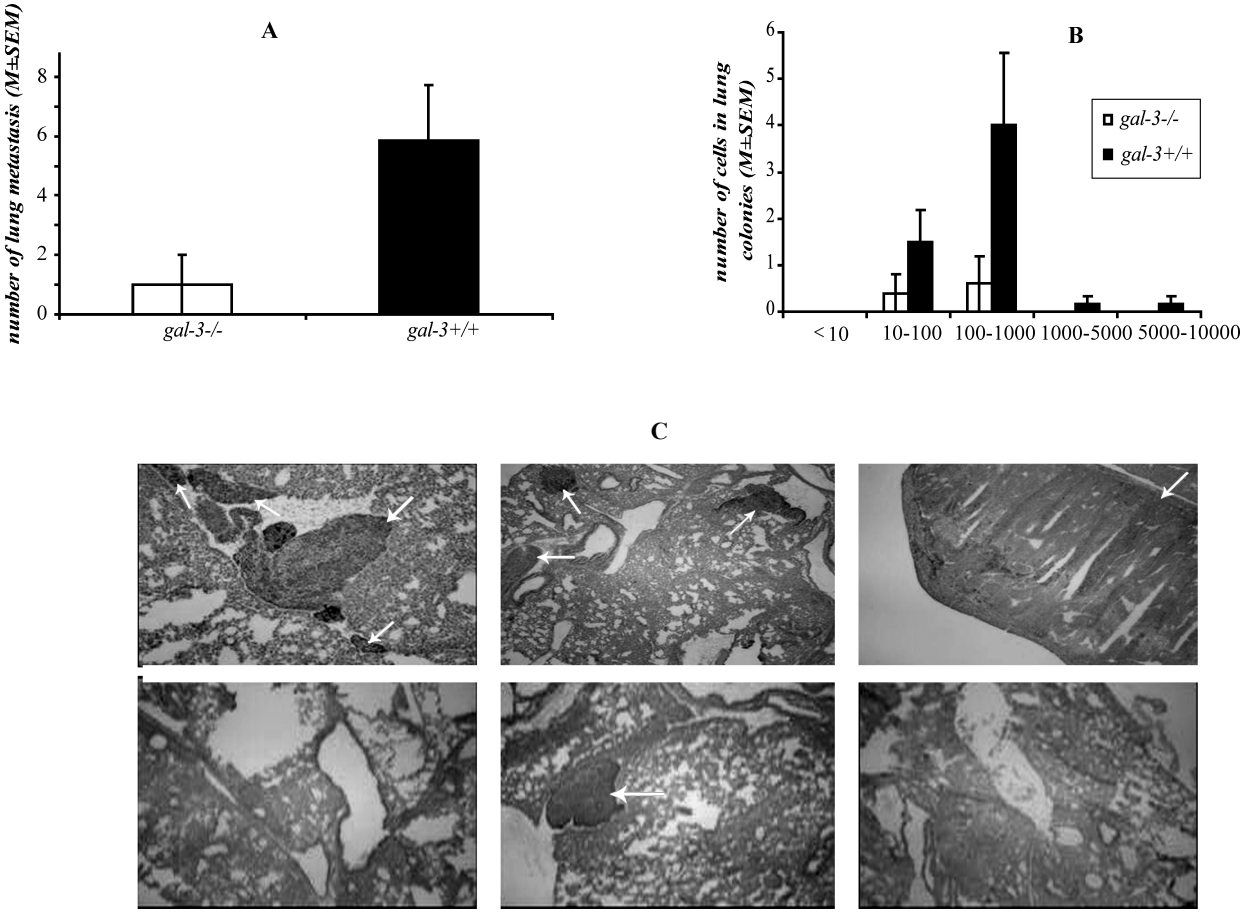

Fig. 1. Lung metastasis and distribution colonies in gal-$3^{-/-}$ and gal-$3^{+/+}$ mice at 21 days after inoculation of 5×10^4 B16F1 per mouse. The number of lung metastasis was significantly lower in gal-$3^{-/-}$ mice (1.00±1.00; *p=0.03) when compared with the gal-$3^{+/+}$ mice (5.83±1.89; Figure 1A). In addition, there was statistically significant differences between gal-$3^{-/-}$ and gal-$3^{+/+}$ mice in the number of malignant cells in the colonies (Mann-Whitney test; p=0.026; Figure 1B). Representative histology of the mouse lung bearing B16F1 melanoma cells in gal-$3^{-/-}$ and gal-$3^{+/+}$ mice (H&E; Figure 1C). Light-microscopic picture showing metastatic colonies in the lung (arrows). Metastatic colonies was not observed in the other parenchymal organs (*data not shown*)

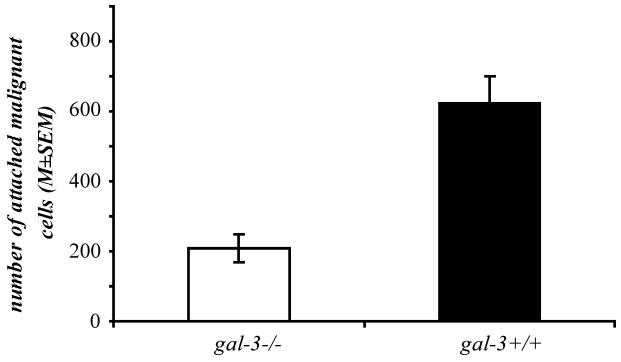

Fig. 2. Adhesion assay: average number of attached B16F1 melanoma cells was significantly higher in the tissue section derived from gal-$3^{+/+}$ mice (623.67±76.55) compared with gal-$3^{-/-}$ mice (Mann-Whitney test; 209.17±39.49; *p=0.001). The mean number of attached malignant cells ±SEM is shown

examined *in vitro* cytotoxic activity of adherent and non - adherent cell populations isolated from spleen of gal-$3^{-/-}$ mice vs. gal-$3^{+/+}$ mice by MTT assay. Mice were divided into 4 groups: gal-$3^{+/+}$ and gal-$3^{-/-}$ mice i.v. injected with $5x10^4$ B16F1 melanoma cells as tumour cells-inoculated groups, and healthy gal-$3^{+/+}$ and healthy gal-$3^{-/-}$ mice, as control groups. Ten days after injection the mice were killed, and spleenic adherent and non- adherent cells isolated from each group were tested for cytotoxicity toward B16F1 cells in a standard MTT assay. We noticed relatively higher but not statistically significant spontaneous cytotoxicity of adherent cells isolated from spleen of untreated gal-$3^{-/-}$ mice compared with gal-$3^{+/+}$ mice, controls (*data not shown*). Remarkably, 10 days after i.v. injection of 5×10^4 B16F1 cytotoxicity of adherent and non- adherent cells populations of gal-$3^{-/-}$ mice was significant higher than in gal-$3^{+/+}$ mice at T:E ratios of 1:2 and 1:4 (p = 0.001; Fig. 3). Herein, the lack of Galectin-3 appear to enhance both innate and specific cytotoxicity in malignant melanoma.

Discussion

A series of experimental studies have been reported that Galectin-3 plays important roles in various processes such as adhesion [24], resistance of metastatic tumour cells to apoptosis (reviewed in 25), neoplastic transformation [26–29], and tumour progression [30], but direct evidence for its role on metastastic process *in vivo* is lacking. The current study was designed to more directly establish the importance of Galectin-3 in melanoma metastasis. Here we demonstrate that lack of Galectin-3 renders mice resistant to melanoma metastasis: *in vivo* study showed that when compared with gal-$3^{+/+}$ mice, gal-$3^{-/-}$ mice exhibited significant resistance to lung colonization of melanoma cells as evaluated by number and size of metastatic colonies (p<0.03). Additionally, there was statistically significant differences between gal-$3^{+/+}$ and gal-$3^{-/-}$ mice in the number of malignant cells in the colonies (p = 0.026). In fact, cellularity of lung colonies in gal-$3^{+/+}$ mice is higher in compared with lung colonies of gal-$3^{-/-}$ mice. Complementary to our findings, Abdel-Aziz HO. et al (31) recently showed that the incidence of lung tumours was significantly low in gal-$3^{-/-}$ mice after intraperitoneal injection of chemical carcinogen such as 4-(methylnitrosamino)-1-(3-pyridyle)-1-butanone. Thus Galectin-3 could be important in lung carcinogenesis as well as in metastasis. In addition, it has been reported that increasing the serum level of Galectin-3 in patients with malignant melanoma, reflect

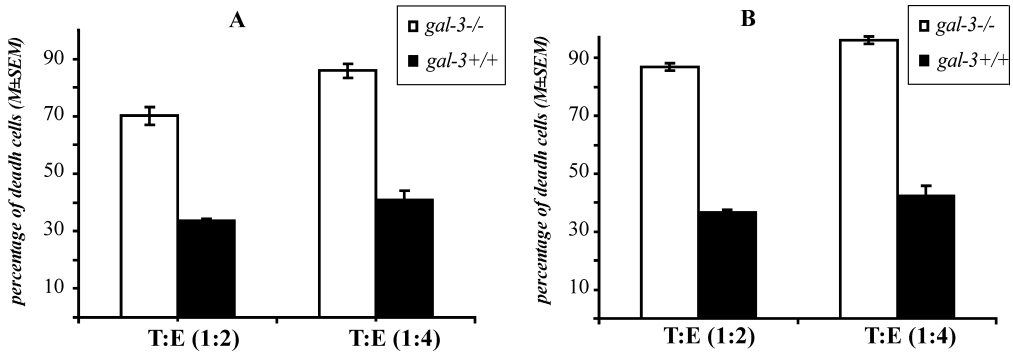

Fig. 3. Cytotoxic responses toward B16F1 melanoma cells in gal-$3^{-/-}$ and gal-$3^{+/+}$ mice immunized with B16F1 cells. Ten days after i.v. injection of 5×10^4 B16F1, cytotoxicity of adherent and non- adherent cells populations of gal-$3^{-/-}$ mice was significant higher than in gal-$3^{+/+}$ mice at T:E ratios of 1:2 and 1:4 (Student's t test; p = 0.001)

biological aspect of tumour behaviour associated with a metastatic phenotype [32, 33].

The adhesive interaction of metastatic tumour cells appears to be obligatory for successful creating of metastatic foci in the distant organs. There is evidence to suggest that Galectin-3 promote tumour cells adhesion *in vitro*. For instance, elevated expression of Galectin-3 markedly enhances tumour cells adhesion to common extracellular matrix proteins [34] and increses the incidence of lung metastasis [35]. This is in agreement with the data presented in this work showing resistance of Galectin-3-defficient mice to melanoma metastasis. In addition, our *in vitro* adhesion assay showed lower capacity of malignant cells to bind onto lung tissue of gal-$3^{-/-}$ mice suggesting that lack of Galectin-3 may contribute to this resistance of mice to melanoma metastasis. This assumption may be supported by findings that Galectin-3 binds specifically the poly-*N*-acetyllactosamine residues of tumour cells [36-38] including melanoma cells [4]. Thus, endothelial Galectin-3 might fuctions to promote tumour cells adhesion to blood vessel wals by interacting with numerous carbohydrate ligands expressed on tumour cells [4, 39, 40].

The resistance mechanism of Galectin-3–deficient mice to melanoma metastasis prompted us to investigate also the role of Galectin-3 in anti -melanoma immunity. We noticed relatively higher but not statistically significant spontaneous cytotoxicity of adherent cells isolated from spleen of untreated gal-$3^{-/-}$ mice compared with gal-$3^{+/+}$ controls (p>0.05). One possible explanation for this observation is that Galectin-3 may act as a downregulator of innate immune response even through alternative activation of macrophages. This is supported by a study of MacKinnon AC. et al. [41] who described that Galectin-3 mediates IL-4 and IL-13-induced alternative activation of macrophages by interacting with CD98. Remarkably in the present study, 10 days after injection of murine melanoma cells, cytotoxicity of adherent and non -adherent cells populations of gal-$3^{-/-}$ mice was significant higher than in gal-$3^{+/+}$ mice (p = 0.001). It is well documented that alternatively activated macrophages (also known as M2 macrophages) by secreting various factors such as interleukin-10, Transforming Growth Factor-в (TGF-в), can suppress immune responses [42-44]. In recent years, it has been reported that Galectin-3 can contribute to tumour cell evasion of immune responses by modulating survival and function of efector T cells. For example, two studies demonstrated that Galectin-3 induces apoptosis of tumour-associated lymphocytes in human melanoma biopsies [16] and tumour reactive CD8+T cells in a mouse model of colorectal cancer [17]. Moreover, Demotte N. et al [45] suggests that Galectin-3 also contributes to tumour immune escapes by inhibiting the function of tumour-specific cytotoxic T lymphocytes (CTLs). Actually, it has been reported that Galectin-3 can promote T cell dysfunction by increasing the distance between the TCR and its co-receptor CD8 in anergic tumour-infiltrating human CTLs [45].

In summing up our results suggest that Galectin-3 contributes to different steps of tumour progression *in vivo* including cell adhesion and tumour -immune escape, suggesting that blockade of Galectin-3 might results in therapeutic benefits.

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ROLE OF GALECTIN-3 IN TUMOUR METASTASIS

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We used metastatic variant of B16 melanoma (B16F1) to study lung colonization galectin-3-deficient (gal-3$^{-/-}$) C57BL/6 mice. *In vivo* study showed that compared with gal-3$^{+/+}$ mice, gal-3$^{-/-}$ mice exhibited resistance to lung colonization of B16F1 melanoma cells (p<0.03). *In vitro* assays showed higher number of attached malignant cells in the tissue section derived from gal-3$^{+/+}$ mice (p<0.001) and tumor specific cytotoxicity of lymphoid cells of tumour inoculated gal-3$^{-/-}$ suggesting that galectin-3 is considered as therapeutic target.

Key words: *Galectin-3, malignant melanoma, metastasis, B16F1.*

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