

HISTOLOGICAL STUDY OF MELANOMA B16F1 INOCULATED IN C57BL /6// BIOU FEMALE MICE TREATED WITH THE FORMULATION CYTOREG®

Rosa De Jesus, Marie Cuervo, Nelson Vicuña-Fernandez, Andrés Osorio, Domingo Stea, David Martucci, Lewis Pozo, William Jiménez.

University of Los Andes, Biology Department, Science Faculty. Merida, Venezuela. Telefax (0274) 2403128. rosadej@ula.ve. cytorexinc@yahoo.com

Abstract

Melanoma is a very aggressive neoplasm. In spite of the scientific advance in the different forms of treatment to combat this disease, such as the use of chemotherapy and radiotherapy, these are not very specific, since they can affect the healthy cells surrounding the melanoma. In this work, we studied the effect of the formulation Cytoreg® on melanoma B16F1, inoculated in female mice C57BL/6//BIOU. The study was performed through the histopathological analysis of the following organs: lung, liver, spleen, heart and skin of the tumor area; histology was performed in addition to the control groups (sick and treated with Intron A). Macroscopically, the presence of metastatic nodules was not observed. The histopathological analysis of the tumors revealed the presence of living tissue located near the functional blood vessels, observing necrotic tissue located near non-functional blood vessels. The percentage of necrotic tissue in the tumors present in each of the groups was: 40% for the EC group, 10% for the animals treated with INT A, and 95% for the Cytoreg® treatment group, leading to conclude that formulation Cytoreg® had an anti-angiogenic effect on melanoma cells.

Key words: neoplasia, Cytoreg®, histopathology, melanoma, mouse C57BL/6//BIOULA

INTRODUCTION

The skin is the largest organ of the body and is involved in the development of melanoma. In it, there are several types of cells, among them: the melanocytes, which are located in the basal layer of the skin, specialized in the synthesis of melanin pigment from tyrosine, which is finally

transported to the upper layers of the skin, where it will absorb visible light and protect living cells from the skin (Shulz 2005). Melanoma is a type of neoplasia (Beers and Berkow 1999), whose origin is focused on melanocytes of the skin (Cabrera and López 2006). The production of melanoma has different causes, including exposure to ultraviolet radiation that causes mutations at the DNA level, leading to the presence of nevi or moles (Acosta et al., 2009). When carrying out a histopathological study of melanoma or of a lesion with suspicious characteristics, uncontrolled growth without melanocyte differentiation with a high production of melanin or absence of melanin (amelaniotic) may be observed in the sample previously treated with histological techniques.

When melanoma of the skin is detected and treated in early stages, there is a high probability of healing (Martí 2003). The treatment of melanoma depends on the depth of the tumor, according to the Clark's level, which is related to the invasion of melanoma in the skin. At level 1, melanoma is confined to the epidermis (melanoma in situ) and has no ability to metastasize; it is treated with surgery (Wong et al., 1993, Sharpless and Das 1998). This can be combined with a therapy post-surgical adjuvant to keep the disease under control (Fitzpatrick 2009). When the tumor is in level 2: Invasion into the papillary dermis, chemotherapy, radiotherapy and interferon are used (Masloski et al., 2008). Levels 3, 4 and 5 are also recognized: level 3 (invasion of the junction of the reticular and papillary dermis); level 4 (invasion within the reticular dermis) and level 5 (invasion within subcutaneous fat). For the treatment of levels 2, 3, 4 and 5, chemotherapy and radiotherapy are used as adjuvant treatments after surgery. However, these are not very specific, since in addition to acting on the cancer cells, they also act on adjacent healthy cells, besides causing secondary effects symptoms, such as nausea and vomiting (Espinosa 1998). Interferon is a cytosine (Abbas 2008) which has a high activity against metastatic melanoma; used as a conventional treatment, it produces apoptosis in different tumor cell lines and in primary tumors (Caraglia et al., 2005); among its actions, it is also found to be antiangiogenic (prevents the formation of new blood vessels from pre-existing vessels), (Raffaella et al., 2002), immune-stimulatory T helper cells, increases the activity of natural killer cells (NK cells) and stimulates the differentiation of hematopoietic cells, such as B and T lymphocytes, dendritic cells and NK (Menon et al., 2004, Yang et al., 2004).

The treatments used against melanoma have been ineffective. In addition, they are not specific enough in their action against cancer cells; so, it is necessary to investigate new forms of melanoma treatment that guarantee the elimination of melanoma cells and the conservation of healthy cells. Faced with this challenge, the Cytorex International Inc. laboratory in Zulia (Venezuela) developed a chemical formulation, which could be considered as an alternative chemotherapy against cancer cells (Kumi-Diaka et al., 2006). This formulation is called Cytoreg®, being defined as an ionic therapy derived from a mixture of strong and weak acids. This is characterized by being a transparent liquid, acid pH, low molecular weight, which does not change its concentration over time, is not photosensitive and can be administered orally, intravenously or topical. The mechanisms of action that are under study have been oriented under the following assumptions: Cytoreg® bombards the membrane of the cells, with the ions of the acids that make it up by altering the electrical charge of the cell (both extra and intracellularly), then taking place an exchange of ions at the molecular level; in this way, Cytoreg® maintains the balance and regulates the metabolism of cells, without producing its death. In a cancer cell where apoptosis is inhibited, the component acids of Cytoreg will provide the necessary ions to compensate the affected cell and induce the metabolism that will activate apoptosis immediately. Additionally, it can act as a stimulant of the immune system. The formulation Cytoreg® has been analyzed in pre-clinical studies conducted in Cancer Research of Europe and USA. Prior to 2001, the formulation was optimized and studies are currently being carried out in animal models in the Vivarium of the Universidad de Los Andes (BIOULA). In the present work, the effectiveness of the formulation Cytoreg® on melanoma caused with cells of the B16F1 line cultured “*in vivo*”, in female mice C57BL/6//BIOU was tested, taking histopathology as a parameter of assessment.

This project was approved by the Ethics Committee of the Vivarium of the University of Los Andes under the Protocol No.: CE BIOULA / 030.

METHODOLOGY

Biological material

We used 30 female mice of the C57BL/6//BIOU line, between 8 and 10 weeks old, produced and

maintained in the Vivarium of the University of the Andes (BIOULA). Fed with commercial Ratarin, treated at a temperature of 120 ° C for one minute and supplied at will, as well as water (sterilized at 120 ° C for 10 minutes). Groups of mice were housed in T2 boxes (26x21x24 cm.) and distributed as shown in the table 1.

Table 1: Experimental groups used in the study

Group	Experimental groups	N ° of animals	N ° of animals per boxes T2
1	Healthy Control (SC)	5	5
2	Sick Control (EC)	10	
3	Doses 0.49 ml / kg (D1C)	10	
4	Intron A (INT A)	5	

Obtaining B16F1 melanoma cells

B16F1 murine melanoma cancer cells were donated by Dr. Juan Luis Concepción, from the Parasite Enzymology Laboratory of the Faculty of Sciences of the Universidad de Los Andes, Mérida (Venezuela). These were extracted from the tumor from a male C57BL/6 mouse. After removing the tumor capsule, the cells were suctioned using a syringe; these cells were diluted in PBS pH 7.4 glycosylated at 0.9%. Subsequently, a mechanical dissociation of the extracted cells was carried out in a Petri dish to eliminate any tissue remnants, using syringes of decreasing caliber, continuously. Once the tissue was removed, the count of the cells to be inoculated was counted, in a volume of 1 to 1 in PBS pH 7.4, glycosylated at 0.9%. The cell count was performed in the Neubauer Chamber.

Inoculation of cancer cells.

Cell transplantation was performed by intramuscular inoculation in the left posterior thigh, in a volume less than 0.1 ml, containing 50,000 murine melanoma cancer cells. This location facilitated effective replication, easy tracking and measurement of tumors. The evaluation time of the tumor growth was performed during 10 post-inoculation days.

Evidence of effectiveness of Cytoreg®

After 10 post-inoculation days of the melanoma cells, the Cytoreg® formulation was orally administered at a dose of 0.49 ml/kg (minimum lethal dose, determined in previous experiments) (Table 1: group DIC). The formulation was administered via voluntary intragastric route. At the same time, INTRON A was started (table 1: INT A group). This was administered intraperitoneally, three times a week with a dose of 66 IU/ μ l dissolved PBS pH 7.4. Prior to the administration of the treatments, hematological tests were performed and the animals were weighed; this evaluation was carried out weekly until the end of the 15 days of treatment. The tests were also carried out on the sick control groups (SC), and the healthy control group (EC).

Applied histological technique

After spending three weeks administering the treatment, the mice were sacrificed in sacrificial chamber with excess enflurane, and a proportion of the heart, lung, spleen and limb affected by the tumor were taken for histopathological evaluation. The histological processing was carried out following the technique proposed by Eynard et al. (2008), using inclusion in paraffin; cuts (5-10 μ m) of tissue were made in microtome and hematoxylin-eosin staining was performed.

RESULTS

Anatomopathological description of the target organs of B16F1 melanoma in the different groups: controls and experimental, and of the tumor

In the macroscopic observation of the organs that are considered target organs of melanoma B16F1, in a general way it can be said that all: liver, lung, spleen and heart, presented a similar appearance in relation to their morphology and their coloration. Among the mice of the group of healthy mice and groups of sick mice, both the control and the treated, in the latter there were not metastatic nodules observed.

Anatomopathological description of the tumor

Figure 1 shows the tumors extracted from the groups of mice treated with the formulation Cytoreg® D1C (1B), and the EC (1A) and INTA (1C) controls. In the tumors of the untreated or sick control (EC) mice and those treated with INTA, light colored areas were observed, while the tumors of the treated with Cytoreg (group D1C) showed colorations from very light grayish to one more color dark that was almost black. In all tumors, the presence of blood vessels feeding the tumor was observed; the shape of the tumors was irregular and their consistency was soft and not very compact when they were removed from the capsule that surrounded them.

Histopathological description of each of the white organs of B16F1 melanoma in the different experimental and tumor groups.

Figure 2 shows the histological sections of the lung, in which it is detailed that both the EC control and the group treated with Cytoreg D1C, presented a healthy parenchyma; however, in the diseased mice treated with intron, pneumonia characteristics are observed, showing a consolidation zone where a normal architecture substitution occurs with the accumulation of inflammatory cells within the specifically neutrophilic alveoli and a thickening of the bronchial septa, a chronic bronchitis of lesser intensity presenting neutrophils. In the rest of the other white organs: spleen, liver and heart, examined histologically, all presented a conserved architecture and without signs of pathology.

Figure 3 shows the melanoma tumor tissue induced in the thigh of the animals treated with the

formulation Cytoreg® and the EC and INT A controls. In these, a clear differentiation of two zones was observed, one basophilic (dark purple coloration) and another eosinophilic (pink). In the tumors of the mice treated with Cytoreg and in the diseased controls and treated with INT A, it could be observed that the living tissue were the melanoma cells, while the necrotic tissue were melanoma cells. Melanoma cells that survived were found around the vessels (small arrow) that fed the tumor, while the rest of the necrotic tissue was tissue away from the vessels (Figures 3A, B, C: long arrow). The percentage of necrotic tissue in each of the groups involved was 40% for the EC group, 10% for the INT A control, and 95% for the D1C treatment group. This percentage of necrosis was determined according to the size of the sample of tissue obtained in each treatment.

Fig. 1. Macroscopic characteristics of melanoma tumor B16F1 of each of the experimental groups: (A) Sick Control; (B) D1C (0.49ml / kg); (C) INT A. control



DISCUSSION.

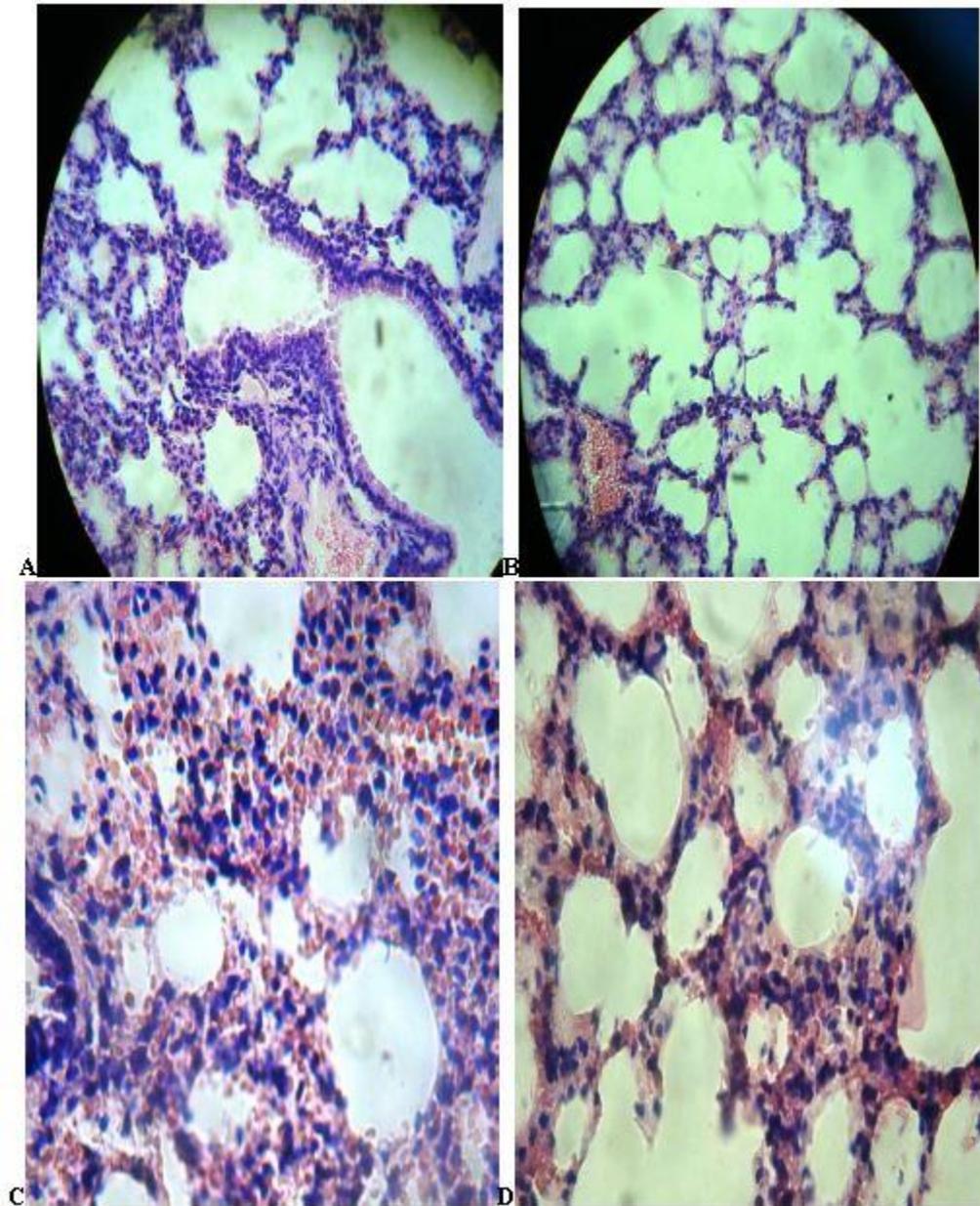
Melanoma, although it represents around 1% of all neoplasms and less than 10% of skin cancers,

is responsible for 75% of deaths from skin cancer. The majority of deaths are due to the spread of the lesion beyond other parts of the same tissue, in neighboring tissues called: invasion; and in distant organs called: metastasis (Chiang and Massagué 2008). This has the ability to metastasize in mainly the lungs and liver, through the invasion of cancer cells on the tissue of these organs; it is therefore important in any study where it is intended to study the effectiveness of any formulation, to observe if there is metastasis of the melanoma cells on the possible white organs of melanoma B16F1, such as: the liver (Latarjet 2004); the spleen, in which it alters its function as a lymphocyte organ (Alvarez et al., 2008); the lung (Cassina et al., 2011), in addition to the heart (Ozyuncun et al., 2006), the latter being very rare; however, they may occasionally occur. The term melanin heart was coined by William Norris in 1850. The metastatic involvement of melanoma in the heart occurs frequently in the myocardium with a rate of 98%, also in the epicardium and in the endocardium with frequency rates of 78% and 73%, respectively (Kuznitzky et al., 2003). In the study performed, as reported in the results, in none of the anatomopathological studied organs there was presence of any metastatic nodule, in any of the groups transplanted with the cells of the melanoma cell line used, line B16F1, corroborating some bibliographic reports, on the characteristic of this line of melanoma of having little metastatic potential at the level of the lungs. This result is strengthened by not finding melanoma cells in the histology performed on these organs.

The melanoma tumor showed a continuous increase in all the mice in which the melanoma cells were transplanted; however, the growth after 10 post-transplant days was lower in the mice that were treated with the formulation Cytoreg®. In the histopathological analysis of the melanoma tumor, areas of blackish coloration were observed in all the mice; these were not as a consequence of the presence of melanocytes that produce a hyperpigmentation, due to the melanin that also gives a gray or blackish color and areas of very clear gray; such coloration, possibly, according to the results of the histological sections of the tumor, was due to the presence of necrotic areas, since the melanoma tumor cells observed in the cuts were of the amelanotic type that is characterized by the lack of pigmentation. These necrotic areas presented different percentages of necrotic tissue, being almost 95% in the mice treated with the Cytoreg formulation, inferring then that this greater percentage of necrotic tissue could occur due to the death of the cells caused by the effect of Cytoreg on the blood vessels. The spaces in which melanoma cells live, were observed were those

found near functional blood vessels, while necrotic tissue, despite having blood vessels, apparently were not functional, a necessary condition for the survival of melanoma cells. In other words, Cytoreg® would be affecting both the blood vessels and the tumor cells.

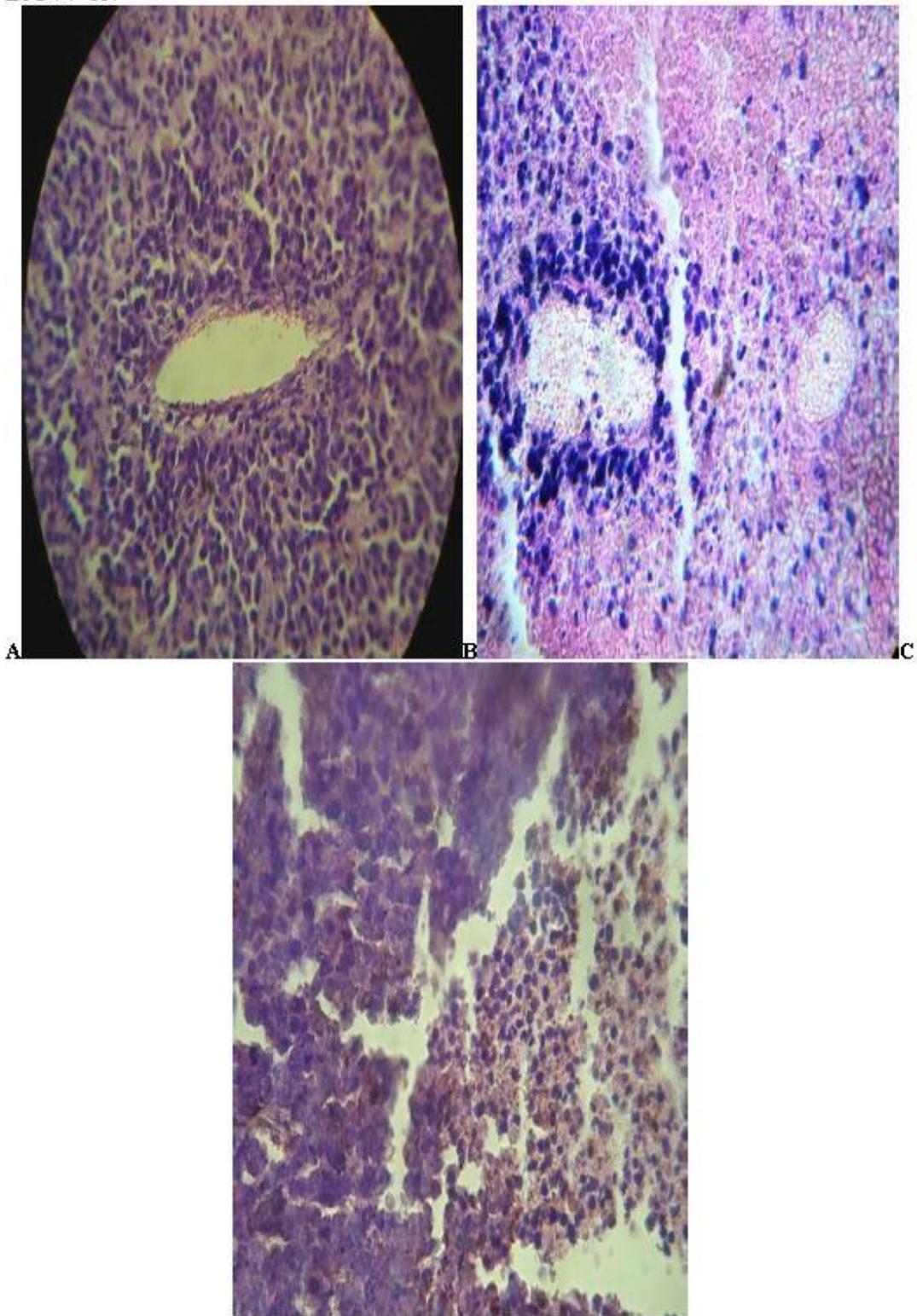
Fig. 2. Histological sections of the lung of the treatment groups (A) EC, (B) D1C, (C) INT A, (D) SC (40X, cross section).



CONCLUSIONS

1. The macroscopic study evidenced the absence of metastatic nodules in white melanoma organs.
2. The histology of possible white organs (lung, liver, spleen and heart) of melanoma B16F1, revealed the absence of metastasis.
3. The histology of the tumors revealed that the group of mice to which the formulation Cytoreg® was administered had a high percentage of necrotic tissue with respect to the controls.
4. The most efficient treatment, according to the results obtained at the histopathological level, was that of the D1C group with 95% necrosis, compared with the EC and INT A controls with 40% and 10% necrosis, respectively.

Fig. 3. Histological sections of the melanoma tumor of the groups of (A) EC, (B) D1C, (C) INT A (40X, cross section).



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