**Interleukin-2 increases intracellular glutathione levels and reverses the growth inhibiting effects of cyclophosphamide on B16 melanoma cells**

Teodoro Palomares, Ana Alonso-Varona*, Antonia Alvarez*, Begoña Castro*, Yolanda Calle* and Pedro Bilbao†

Department of Surgery, Radiology and Physical Medicine and *Department of Cell Biology and Morphological Sciences, School of Medicine and Dentistry, University of the Basque Country, Leioa, Vizcaya, and †Department of Radiation Oncology, Txagorritxu Hospital, Vitoria, Spain

(Received 12 August 1996; accepted in revised form 12 November 1996)

Glutathione (GSH) plays an essential role in the metabolism of melanoma. As changes in intracellular GSH content can modify the processes of cell proliferation and detoxification, this could determine the therapeutic response to some cancer treatment strategies. The purpose of this study was to test the effects of treatment with interleukin-2 (IL–2), alone and in combination with cyclophosphamide (CY), on survival of mice bearing B16 melanoma liver metastases, and to determine the influence of these therapeutic agents on the GSH metabolism of B16 cells. In the \textit{in vivo} test system, B16 melanoma liver metastases were induced in C57BL/6 mice which were subsequently treated with IL–2, CY and CY plus IL–2. Survival time was used to determine the response to treatment. In the \textit{in vitro} system, we evaluated the effects of IL–2, acrolein (an active metabolite of CY responsible for GSH depletion) and acrolein plus IL–2 on GSH levels and proliferation of B16 melanoma cells. Results indicated that, \textit{in vivo}, all treatments increased mouse survival times with respect to control mice. However, the addition of IL–2 to CY therapy decreased survival time compared with treatment with CY alone. \textit{In vitro}, whereas acrolein produced a GSH depletion and inhibited B16 cell proliferation, IL–2 increased GSH content and cell proliferation rate compared with untreated cells. Moreover, addition of IL–2 to cells preincubated with acrolein increased GSH levels and proliferation with respect to acrolein alone. In summary, the data suggest that GSH plays a critical role in the growth-promoting effects of IL–2 on B16F10 melanoma cells and in the antagonistic effect of IL–2 on CY inhibitory activity on these tumor cells.

**Keywords:** B16 melanoma, cyclophosphamide, glutathione, interleukin 2

**Introduction**

Metastatic melanoma cells can vary widely in their growth rates, degree of pigmentation and morphology [1]. Since the processes of differentiation, growth and melanization are intimately related in melanoma cells, the possible association between these biological parameters and metastasis formation has been extensively investigated, although inconsistent results have been described [2–4]. The level of intracellular glutathione (GSH) could be a common factor involved in this association. This ubiquitous tripeptide thiol plays an essential role in the particular metabolism of melanoma. GSH metabolism is required in a variety of cellular
homeostatic and regulatory functions including DNA and protein synthesis [5], the biosynthesis of melanin [6,7] and scavenging of the free radicals generated during melanogenesis [8,9]. Moreover, it has been shown that melanoma cells have significantly higher GSH contents than nontumorigenic melanocytes, a finding which has also been related to the inherent drug resistance of melanoma [10]. In contrast, GSH depletion impairs the viability of melanoma cells and reduces the metastatic spread of these tumor cells when transplanted in vivo [11].

Several biological agents can modulate melanin production and the metastatic potential of melanoma cells, including melanocyte-stimulating hormone [12] and cytokines such as interferon-gamma [13] and interleukin 1 (IL–1) [14]. Cytokines are believed to play an important role in the immune regulation of the skin and recently a network of interacting cytokines has been proposed [14]. In this regard, the expression of several cytokines such as IL–1 and IL–2 have been demonstrated in normal cultured melanocytes [15] and murine [16] and human [15,17] melanoma cell lines.

IL–2 is a lymphokine that has been used in the immunotherapeutic treatment of malignant melanoma, both alone [18] or in combination with cytotoxic drugs such as cyclophosphamide (CY) [19]. Several reports have demonstrated the relationship between these agents; IL–2 and CY, on the one hand, and intracellular GSH levels in different types of cells, on the other. Whereas IL–2 increased GSH content in lymphocytes [20], CY decreased intracellular GSH levels in normal and tumor cells [21], essentially due to acrolein, one of its active metabolites.

Recently, we have shown that the addition of IL–2 to CY therapy of mice bearing B16 liver metastases could reduce the inhibitory effects of CY, increasing tumor growth with respect to CY alone [23]. This study supported the notion of a growth-promoting effect of IL–2 on B16 cells, which may be exerted through specific cell surface receptors [16]. These data suggest that IL–2 and CY might exert antagonistic effects on intracellular GSH levels of B16 melanoma cells, which could influence their proliferation and melanization.

To test this hypothesis, in the present study we evaluated the in vivo effects of exogenous IL–2, alone and in combination with high-dose CY, on the survival of mice bearing B16 melanoma liver metastases. Also, in studies in vitro we evaluated the effects of IL–2, alone and in combination with acrolein, on GSH levels and on the proliferation of B16 melanoma cells.

Materials and methods

Animals and tumor cells
Female C57BL/6 mice (6–8 weeks old) were used in all experiments. The mice were purchased from Iffa Credo Laboratories (L’Arbreole, France). They were given food and water ad libitum, and kept on a 12 h day/night cycle.

The B16F10 melanoma cell line was maintained as a monolayer culture in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO) in a humidified atmosphere (5% CO₂, 95% air) at 37°C.

Cell culture
Exponentially growing cell cultures were used in all experiments. After trypsinization and centrifugation, the cell pellet was resuspended in complete medium plus FCS and the cells were enumerated with a Coulter counter. Viability, as determined by trypan blue exclusion, ranged from 95 to 98%. For certain assays, B16F10 melanoma cells were seeded in 96-well microplates at a density of 10³ cells/well in 200 μl of growth medium plus 10% FCS and were incubated at 37°C overnight prior to drug exposure.

Drugs
Cyclophosphamide (CY) (Genoxal, Funk) was dissolved in sterile distilled water and was administered intraperitoneally (i.p). Dosages used were adjusted for body weight. The maximum tolerated dosage (MTD) for a single cycle of treatment was determined to be 300 mg/kg. Recombinant human interleukin 2 (rIL–2) was obtained from Boehringer Mannheim (Germany) with a biological activity of 2 × 10⁶ U/mg. It was reconstituted in water, diluted in calcium and magnesium-free Hank’s balanced salt solution (HBSS) and injected i.p.

GSH determination
One, 4, 24 and 48 h after drug exposure, the medium from cultures was removed and the cells were washed twice with PBS. For GSH assay, the cells were stained with 20 μM monochlorobimane (Molecular Probes, Eugene, OR) for 60 min at 37°C [25]. The measure of intracellular GSH content was proportional to their fluorescence intensity determined using the CytoFluor–2350 system (Millipore Co., Bedford, MA) at 360/40 nm excitation with a 460/40 nm emission filter, at high and intermediate sensitivity settings. Corrections for autofluorescence were made by subtracting fluorescence measured in unstained cells to obtain a normalized fluorescence.