BIOCELL
2012, 36(2): 91-95

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Modulation of IL-10/IL-10R expression by mafosfamide, a derivative of 4-hydroxycyclophosphamide, in a rat B-cell lymphoma

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Key words: metastasis, cyclophosphamide, cell proliferation

ABSTRACT: We have already shown that IL-10 plays an important role in immunosuppression and metastatic dissemination in the rat B-cell lymphoma L-TACB model. It was suggested that the up-regulation of IL-10 production and IL-10 receptor (IL-10R) expression would be part of the transition from primary tumor to metastatic phenotype and that IL-10, besides its immunosuppressive activity, may act as a growth factor for metastatic L-TACB cells. The treatment of L-TACB-bearing rats with a single low-dose cyclophosphamide decreased IL-10 production, reverted immunosuppression and induced the immunologic rejection of tumor metastasis without any effect on primary tumor growth. Our current aim was to investigate the effects of cyclophosphamide on the expression of IL-10 and IL-10R on primary and metastatic L-TACB cells. Considering that cyclophosphamide is a prodrug, we used mafosfamide, a compound that yields *in vitro* the same active metabolites as cyclophosphamide does *in vivo*. Mafosfamide induced down-regulation of IL-10 production and IL-10R expression on metastatic cells and, concomitantly, inhibited metastatic cell proliferation. We suggest that mafosfamide would inhibit the regulatory loop mediated by the IL-10/IL-10R system and, as a consequence, metastatic cell proliferation. These results may have a considerable impact on the design of new therapies for metastatic lymphomas.

Introduction

During tumor progression, cancer cells change (enhance or decrease) the expression of several growth factors, cytokines, and their specific receptors, as well as other soluble factors or cell membrane receptors, in order to grow autonomously and to acquire competence to metastasis (Pals *et al*., 2007). Interleukin-10 (IL-10) and its receptor (IL-10R) have been implicated in progression and metastasis of different tumor types, including lymphoma. It was shown that the molecular signature of mantle cell lymphoma (MCL), a non-Hodgkin lymphoma, included overexpression of IL-10R, and that IL-10 was able to sustain tumor cell survival and proliferation (Visser *et al*., 2000). On the other hand, overproduction of IL-10 is strongly associated with the immunosuppression frequently observed in tumor-bearing hosts and it could be respon-

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Received: May 2, 2012. Revised version received: July 14, 2012. Accepted: July 17, 2012.

sible for the tumor escape from immune rejection (Salazar-Onfray *et al*., 2007).

We have proposed that the IL-10/IL10R system represents a promising therapeutic target for malignant lymphoma (Croci *et al*., 2007). In our current experiments we used the rat B-cell lymphoma L-TACB model, in which IL-10 appears to play a pivotal role in immunosuppression and metastatic dissemination (Rico *et al*., 2005), and its inhibition or activity modulation constitutes an important therapeutic option (Matar *et al*., 2002). Our previous results in this lymphoma model showed a differential effect of cyclophosphamide on primary tumor or metastasis growth: a single low-dose cyclophosphamide inhibited metastasis development without any effect on primary tumor growth (Matar *et al*., 1995). This antimetastatic action of cyclophosphamide was mediated by immunomodulation since this effect could be adoptively transferred by immune cells of cyclophosphamide-treated tumor-bearing rats and the same treatment did not induce any effect in immunodeficient nude mice (Matar *et al*., 1998). We showed that IL-10 was the main factor responsible for the induction and development of immunosuppression in L-TACB-bearing hosts and that the antimetastatic immunomodulatory effect of cyclophosphamide was mediated by a reduction in IL-10 levels produced by T-lymphocytes (Matar *et al*., 2000; Matar *et al*., 2001). In fact, cyclophosphamide induced a Th2/Th1 switch and increased the proliferation rate of spleen cells (Matar *et al*., 2002). Also, we have shown that L-TACB cells from metastatic nodes produced high amounts of IL-10 and expressed higher IL-10 receptor (IL-10R) levels than L-TACB cells from primary tumors, which did not produce IL-10 at all (Rico *et al*., 2005). Also, the addition of IL-10 *in vitro* induced an increase in proliferation rate only on metastatic cells (Matar *et al*., 2002). It was suggested that the up-regulation of IL-10 production and IL-10R expression would be part of the transition from primary tumor to metastatic phenotype and that IL-10, besides its immunosuppressive activity, may act as a growth factor for metastatic L-TACB cells. Altogether, these results suggested that, in addition to its immunomodulatory activity, cyclophosphamide may also be able to regulate the secretion and/or activity of IL-10 produced by metastatic cells.

Thus, our current aim was to investigate the *in vitro* effects of active metabolites of cyclophosphamide on the expression of IL-10 and its specific receptor IL-10R on both primary tumor and metastatic L-TACB cells.

Materials and Methods

Drugs

Considering that cyclophosphamide is a prodrug, we used mafosfamide (Baxter Oncology GmbH, Germany), a compound that yields *in vitro* the same active metabolites as cyclophosphamide does *in vivo* (Niemeyer *et al*., 1984). Solutions of 2.5, 5, 10 and 20 µM mafosfamide were prepared in RPMI-1640 culture medium (Gibco BRL, Gaithersburg, MD, USA).

Tumor

L-TACB is a poorly differentiated B-cell lymphoma that arose spontaneously in an inbred *e* rat (Calderari *et al*., 1991). It is maintained by serial subcutaneous grafting by trocar of 1 mm^3 tumor fragments (approximately 10^6 cells) in syngeneic rats. When L-TACB is injected s.c., metastases occur exclusively in regional (axillary or inguinal) homolateral or contralateral lymph nodes in 20–30% of the animals.

Preparation of tumor single-cell suspensions

To obtain single-cell suspensions from primary tumor and metastasis, *e* rats were implanted subcutaneously with L-TACB and 21 days later primary tumors and lymph node metastases were excised and cell suspensions were obtained by mechanical disruption in RPMI-1640 culture medium. At this time of tumor progression, hematoxylin–eosin staining showed that normal lymphoid tissue in an organized form was completely replaced by tumor tissue in lymph nodes, and that the only non lymphoid cells appearing could be assigned to nervous tissue and blood vessels (data not shown).

Cell proliferation

Primary and metastatic L-TACB cells were incubated at $1x10^4$ per well in 100 µl RPMI-1640 culture medium with 10% fetal calf serum (FCS) (Natocor, Córdoba, Argentina) containing 0, 2.5, 5, 10 or 20 µM mafosfamide, at 37ºC and 5% CO_2 for 48 h in 96-well plates. Cell proliferation was determined by the colorimetric WST-1 assay (Roche Diagnostics, Mannheim, Germany) following manufacturer instructions. Determinations were done in quadruplicate.

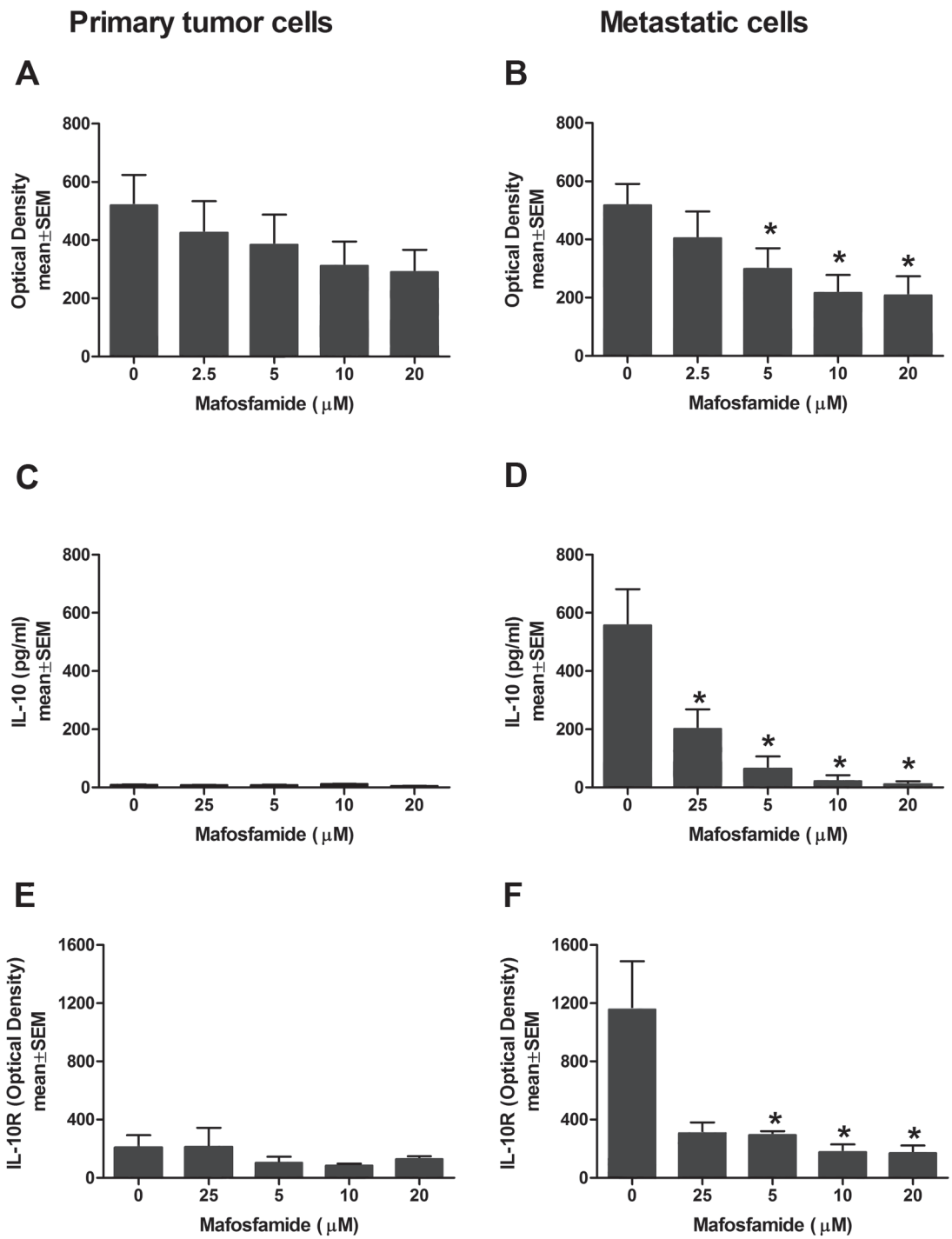

Figure 1. Effect of mafosfamide on cell proliferation, IL-10 production and IL-10R expression of primary tumor cells (1A,1C,1E, respectively) and metastatic cells (1B,1D,1F) of B-cell lymphoma L-TACB. ANOVA: (B) mafosfamide 0 μM vs 5, 10 or 20 μM, $P<0.05$; (D) mafosfamide 0 μM vs 2.5, 5, 10 or 20 μM, $P<0.05$; (F) mafosfamide 0 μM vs 5, 10 or 20 μM, $P<0.05$.

Quantification of IL-10

The concentration of IL-10 was measured in conditioned media from primary tumor or metastatic L-TACB cells by a sandwich ELISA assay. Five x 10^5 cells of each cell type suspended in 5 ml of RPMI-1640 culture medium supplemented with 10% FCS and mafosfamide (0-20μM) were seeded in 25 cm^2 culture flasks. After 48 h incubation at 37ºC and 5% CO_2, culture supernatants were collected and centrifuged. The cell-free conditioned media were aliquoted and stored at -20ºC until used. The ELISA sandwich technique, that uses two different antibodies against different epitopes of IL-10, was carried out according to the instructions provided in the kit (OptEIA™ Rat IL-10 Set; Pharmingen, San Diego, CA, USA).

Expression of IL-10 receptor (IL-10R)

The effect of mafosfamide on IL-10R expression on primary tumor or metastatic cells was determined by CELISA (cellular ELISA) assay. Cells were pre-incubated with 0, 2.5, 5, 10 or 20 μM mafosfamide during 48 h and then fixed at 5 x 10^5 cells/well onto 96 wells microtiter plates with 0.5% formaldehyde-buffer for 18 h at 37ºC. Then, recombinant IL-10 (rIL-l0) at a saturating concentration (5000 pg/ml) was added. The plates were incubated 2 h at room temperature, washed, blocked and then incubated for 1 h with anti-IL-l0-biotin/streptavidin-peroxidase conjugate. After the addition of tetramethylbenzidine and H_2O_2 and 30 min incubation at room temperature in the dark, the reaction was stopped with H_2SO_4. The optical density was determined at 450-570 nm. All samples were assayed in quadruplicate.

Statistical analysis

ANOVA was performed using GraphPad Prism® version 3.03 (GraphPad Software, San Diego, CA, USA). Differences between groups were considered significant when $P < 0.05$.

Results

Effect of mafosfamide on primary and metastatic L-TACB cell proliferation

Mafosfamide did not show any effect on primary tumor cells proliferation (Fig. 1A). In contrast, metastatic L-TACB cells were sensitive to the antiproliferative effect of mafosfamide in a dose-dependent manner, from 5 to 20 μM, when compared to cells incubated without mafosfamide ($P<0.05$) (Fig. 1B).

Effect of mafosfamide on IL-10 production

Quantification of IL-10 showed non-detectable levels in conditioned media from primary tumor cells (Fig. 1C), while the IL-10 levels in conditioned media from metastatic cells were higher in control cells than in cells treated with different doses of mafosfamide ($P<0.05$) (Fig. 1D). Considering that mafosfamide exerts a dose-dependent antiproliferative effect on metastatic cells (Fig. 1B), IL-10 levels were normalized to cell number for each mafosfamide concentration. In this condition, we also observed a mafosfamide-induced dose-dependent reduction of IL-10/cell (data not shown).

Effect of mafosfamide on IL-10R expression

The results showed that primary tumor cells expressed low levels of IL-10R, which were not significantly modified by either dose of mafosfamide (Fig. 1E). On the contrary, metastatic L-TACB cells expressed higher levels of IL-10R with respect to primary tumor cells ($P<0.05$). Moreover, incubation with mafosfamide decreased IL-10R expression on metastatic L-TACB cells at doses of 5 to 20 μM ($P<0.05$) (Fig. 1F).

Discussion

In this rat lymphoma model, as well as in human lymphomas, IL-10 plays a central role in the inhibition of the antitumor immune response as well as a growth factor for metastasis. Nowadays, high levels of IL-10 are considered as significant unfavorable prognostic factor and an International Prognostic Score-independent risk factor for treatment failure in advanced Hodgkin lymphoma (Matar *et al*., 2001; Rautert *et al*., 2008). We have shown that the treatment of lymphoma-bearing rats with a single low-dose cyclophosphamide inhibited metastasis development, and that this effect was mediated by cyclophosphamide-induced immunomodulation, mainly through a reduction in IL-10 levels (Matar *et al*., 1998; 2000). We have also indicated that IL-10 may act as a growth factor for metastatic tumor cells in addition to its immunosuppressive activity (Rico *et al*., 2005). Similar results were obtained in leukemic mantle cell lymphoma (Visser *et al*., 2000), B16 mouse-

melanoma model (García-Hernández *et al*., 2002), and freshly resected primary tumor and metastases from melanoma patients (Yue *et al*., 1997). As far as we know, we demonstrated here a novel effect of mafosfamide on the expression of IL-10 and its specific receptor on lymphoma cells. We observed that mafosfamide, a derivative of 4-hydroxycyclophosphamide, induced down-regulation of IL-10 production and IL-10R expression on metastatic tumor cells and, concomitantly, inhibited metastatic cell proliferation. Taking into account that IL-10 induces *in vitro* metastatic cell proliferation (Matar *et al*., 2002), and that metastatic cells simultaneously express IL-10R and produce IL-10, an autocrine and/or paracrine loop would take place, thus stimulating, either directly or indirectly, metastatic cell proliferation. We suggest that mafosfamide would inhibit the regulatory loop mediated by the IL-10/IL-10R system and, as a consequence, it would inhibit metastatic cell proliferation. Considering that mafosfamide yields the same active metabolites as cyclophosphamide in equimolar concentrations, it can be hypothesized that cyclophosphamide *in vivo* would produce a similar effect than mafosfamide *in vitro*. Then, the effects of IL-10 we have previously observed in L-TACB-bearing rats, namely, immunosuppression and induction of metastatic cells proliferation, could have been abrogated when animals were treated with antimetastatic low doses of cyclophosphamide. These results may have considerable impact on development of new therapies for metastatic lymphomas in view that a single low-dose of cyclophosphamide, a treatment devoid of toxicity, would act inhibiting at least two mechanisms contributing to malignant growth and progression: escape to immune rejection and autonomous growth of metastatic tumor cells.

Acknowledgements

This work was supported by a grant from Alberto J. Roemmers Foundation to Pablo Matar.

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