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Bryostatin 1 enhances lymphokine activated killer sensitivity and modulates the β^1 integrin profile of cultured human tumor cells

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Bryostatin 1 interferes with protein kinase C (PKC) signaling which is involved in the activation of human and murine cytotoxic T lymphocytes, and in the growth and differentiation of tumor cells. Bryostatin 1 has immunomodulating and antitumor properties as demonstrated by preclinical and clinical studies. Here we report that bryostatin 1 increases the susceptibility to lymphokine activated killers and modifies the pattern of β^1 integrin expression of human tumor cells. On the basis of these results the use of bryostatin 1 in combination with immunostimulating cytokines such as interleukin-2 in the treatment of human cancer is suggested.

Key words: β^1 integrins, bryostatin 1, human tumor cells, lymphokine activated killer.

Introduction

New approaches to the treatment of human cancer can be derived by the use of compounds which selectively interfere with tumor cell signaling and tumor–host interactions.[1] Protein kinase C (PKC) represents an important target for such antitumor approaches.[2]

Bryostatin 1, a compound which has been derived from the marine bryozoan *Bugula neritina*,[3] has been found to bind PKC, determining a rapid increase of its enzymatic activity, followed by depression after prolonged exposure.[4]

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Preclinical studies have shown that bryostatin 1 is able to induce cell differentiation[5,6] and growth inhibition[7] on human tumor cell lines *in vitro*. Immunomodulating effects of bryostatin 1 have been demonstrated by the induction of cytokine release, and the activation of murine and human cytotoxic T lymphocytes (CTLs).[8–12] The above findings prompted the use of bryostatin 1 as an anticancer agent in phase I clinical trials.[13] An important finding from such clinical studies is the increase of the serum levels of tumor necrosis factor (TNF)-α and interleukin-6 (IL-6) induced by bryostatin 1 in treated patients, suggesting that such a compound could also have biomodulatory properties in humans. Thus, a novel antitumor approach based on the combined use of immunostimulatory cytokines and bryostatin 1 appears feasible.

In this regard the effect of bryostatin 1 on tumor cell sensitivity to activated lymphocytes is of interest. It is now becoming clear that tumor-specific events, such as the occurrence of cell populations resistant to cell-mediated cytotoxicity, play an important role in determining the clinical response to immunotherapy.[14] It has been observed that recombinant interleukin-2 (rIL-2) often fails to exert any antitumor activity even in the presence of strong immunomodulatory effects.[15,16] Moreover, tumor cells from hematologic[17] and solid malignancies[18] appear heterogeneous in their sensitivity to natural killer (NK)/lymphokine activated killer (LAK) effectors.

We have evaluated whether the exposure of human tumor cells to bryostatin 1 concentrations within the nanomolar range could affect their sensitivity to LAK-mediated cytotoxicity in a standard ^{51}Cr-release assay. We have also studied the effects of bryostatin 1 on the β^1 integrin profile of tumor

cells[19] These integrin receptors might play an important role in determining the invasive potential and also the sensitivity of several types of human tumors to cell-mediated cytotoxicity.[18] High expression of such molecules could in fact increase the surface binding of extracellular matrix proteins and indirectly enhance the effector–target interactions. Cytotoxic lymphocytes might in turn express a number of such receptors or their ligands on the cell membrane.[19]

Materials and methods

Cell cultures

Human melanoma GLL-19 cells, kindly provided by Dr J Guardiola (Istituto Internazionale di Genetica e Biofisica, Naples, Italy) and lung adenocarcinoma A549 [purchased from American Tissue Culture Collection, (ATCC), Rockville, MD] were, respectively, grown in RPMI 1640 medium and Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and antibiotics, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES). Colon adenocarcinoma LoVo cells, kindly provided by Dr G Parmiani (Istituto Nazionale dei Tumori, Milan, Italy), were grown in HAM'S F12 medium, with 10% heat-inactivated FBS, 2 mM L-glutamine and antibiotics, 20 mM HEPES. Breast adenocarcinoma T47D and MDA-MB468, purchased from ATCC, were grown in EMEM medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and antibiotics, 20 mM HEPES and 10 IU/ml insulin.

LAK generation from human peripheral blood lymphocytes (PBL)

PBL obtained by Ficoll-Hypaque separation were incubated in RPMI 1640 medium with 10% FBS, L-glutamine and antibiotics. IL-2 (1000 IU/ml; Cetus, Emeryville, CA) was subsequently added and the PBL were incubated at 37°C for 18 h. IL-2 treatment of human PBL, under these conditions, induces LAK activity which appears mostly due to activated NK cells.[21]

^{51}Cr-release cytotoxicity assay

Human tumor cells were seeded in 96-multiwell plates (Falcon) and incubated with ^{51}Cr (chromic chloride, 37 MBq, 1.00 mCi; Amersham, Amersham, UK) (30 000–50 000 c.p.m./well) for 30 min at 37°C, after incubation in complete medium in the presence and absence of different concentrations of bryostatin 1. After washing with PBS without Ca^{2+} and Mg^{2+}, LAK cells were added to the dishes at different effector:target ratios. Tumor cells and immune effectors were incubated at 37°C for 4 h and 100 ml supernatant medium from each of triplicate samples was then counted in a gamma counter (Beckman). Results were expressed as percentage specific release:

$$\text{specific release \%} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

using ^{51}Cr (c.p.m.) in supernatant from the experimental incubation, spontaneous ^{51}Cr release from target cells without lymphocytic effectors and total ^{51}Cr from the controls. The SEM values of quadruplicate counts were less than 10%.

Monoclonal antibodies (mAbs)

mAbs recognizing different α chains of human β^1 integrins were obtained from the following sources: anti-α^2 (CD49b) integrin mAb IOP49b, anti-α^4 (CD49d) integrin mAb IOP49d (Immunotech, Marseille, France); anti-α^5 (CD49e) integrin mAb SAM-1 (CLB, Amsterdam, The Nederlands); anti-α^6 (CD49f) integrin mAb 4F10 (Serotec, Oxford, UK).

Live cell radioimmunoassay (RIA) of surface β^1 integrins

Human tumor cells were harvested from subconfluent cultures and seeded in 96-multiwell plates at a density of 5×10^3/well. The cells were then exposed for 24 h to 10 nM bryostatin 1. After overnight incubation in complete medium in the presence and absence of bryostatin 1, the growth medium was removed and 100 µl of 5% BSA (w/v) in DMEM and 0.08% (w/v) sodium azide were added to each well. After 60 min of incubation at 37°C, the medium was removed and cells were washed with DMEM containing 5% BSA and 0.08% sodium azide. The 50 µl of the appropriately diluted anti-β^1 integrin mAbs were added to each well. After incubation for 3 h at 4°C the mAbs were removed and cells were washed twice with PBS/BSA 5% (w/v). Then 75 000 c.p.m. in 50 µl of ^{125}I-labeled sheep

anti-mouse IgG was added to each well for 60 min at 37°C. Following incubation, cells were washed three times with PBS/BSA 5% (w/v) and 50 µl of 2 N NaOH was added to each well, adsorbed with a cotton swab and the radioactivity counted in a Beckman model gamma-counter. The background from the well that received only buffered DMEM was approximately 100–250 c.p.m., and was subtracted from that of the wells exposed to anti-β^1 integrin mAbs. The standard deviations of means of quadruplicate counts were less than 10%.

Results

Bryostatin 1 increases LAK sensitivity of human tumor cell lines

The effects of bryostatin 1 on the LAK sensitivity of human tumor cells were evaluated by means of a panel of continuous cell lines representing different tumor phenotypes encompassing colon adenocarcinoma (LoVo), lung adenocarcinoma (A549), breast adenocarcinoma (T47D, MDA-MB468) and melanoma (GLL-19). We have found that bryostatin 1 increases the sensitivity of all these cell lines to LAK, but some differences were observed in the timing and dose dependency of such effects (Figure 1). The maximal increase in LAK cytotoxicity was observed after exposure to 10 nM on LoVo and A549, 1 nM on T47D, and 0.1 nM bryostatin 1 on MDA-MB468 and GLL-19 cell lines. In all cell lines these effects always occurred within 48 h from the beginning of the exposure to bryostatin 1, followed by a decrease for longer times (Figure 2 and data not shown). The maximal effect was recorded at 12 h on LoVo and GLL-19, and at 24 h on the other cell lines (Figure 2). In these conditions the viability of tumor target cells was not affected by bryostatin 1 exposure as evaluated with the Trypan Blue exclusion dye assay. A slight decrease (10–25%) of cell proliferation was, however, found on all cell lines after exposure to 10 and 1 nM bryostatin 1 (data not shown).

Bryostatin 1 modulates surface expression of β^1 integrins

We have evaluated by a live-cell RIA the effects of bryostatin 1 on cell surface expression of α chains of β^1 integrins on LoVo and A549 cells. The exposure of LoVo cells to 10 nM bryostatin 1 for 24 h determined an about 0.5-fold increase of α^2 (CD49b), α^5 (CD49e) and α^6 (CD49f) immunoreactivity, and induced the expression of α^4 (CD49d) which was not detectable on control cells (Figure 3, left panel). Conversely, after 24 h exposure to 10 nM bryostatin 1 no significant changes of cell surface levels of α^2 (CD49b), α^5 (CD49e) and α^6 (CD49f) integrin chains were detectable, while α^4 (CD49d) showed a consistent reduction on the A549 cell line (Figure 3, right panel). Tumor cell exposure to bryostastin 1 concentrations capable of up-regulating the sensitivity to LAK-mediated cytotoxicity did not result in a similar pattern of β^1 integrin expression in the two cell lines. Different mechanisms therefore appear to be involved in the up-regulation of A549 cell sensitivity to activated immune effectors induced by bryostastin 1.

Discussion

Bryostatin 1 is a promising new drug which determines tumor regression and induction of IL-6 and TNF-α *in vivo*.[13] These clinical observations, together with the immunomodulating properties of this compound, suggest that therapeutic approaches based on the use of bryostatin 1 in combination with cytokines such as recombinant interferon-α and rIL-2 should be explored in preclinical and clinical studies.

We have investigated whether the sensitivity to LAK and the surface expression of β^1 integrins could be modulated by bryostatin 1 on cultured human tumor cells. We have demonstrated that bryostatin 1 at nanomolar concentrations induces an increased sensitivity to LAK cells in all of the cell lines. At these concentrations, bryostatin 1 is also capable of determining a 10–25% growth inhibitory effect which occurs in the absence of toxicity. We have also found that the β^1 integrin profile at the cell surface of colon cancer LoVo and lung cancer A549 is modulated by bryostatin 1. Notably, the expression of α^4 chains, which is absent on untreated controls, can be induced by 24 h exposure of LoVo cells to 10 nM bryostatin 1. Nevertheless, we did not find a homogeneous pattern of β^1 integrin modulation in LoVo and A549 cell lines. Therefore, these results exclude a correlation between these events and the sensitization to LAK cytotoxicity induced by bryostatin 1. Moreover, changes in class I MHC antigen expression were not induced by bryostatin 1 at these experimental conditions (data not shown). Mechanisms other than β^1 integrin modulation and class I MHC changes are likely to be involved in the up-regulatory effects of bryostatin 1 on the LAK sensitivity of human tumor cells.

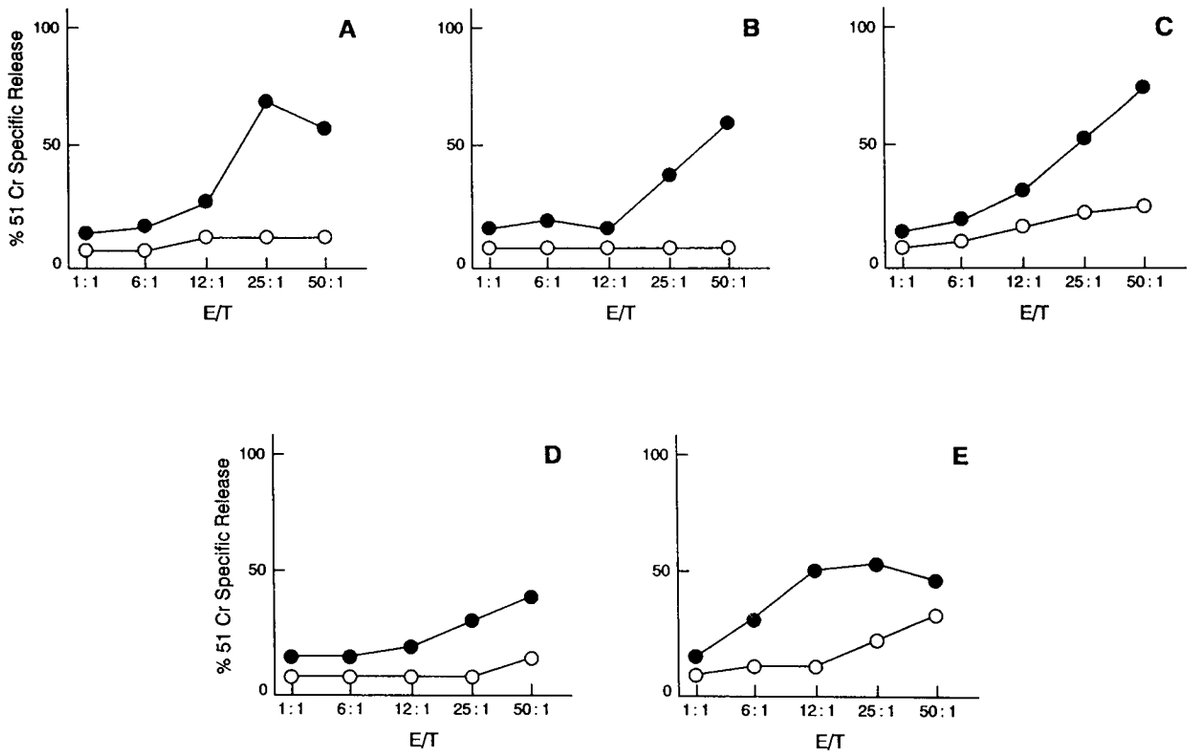

Figure 1. Effects of bryostatin 1 on the LAK sensitivity of A549 (A), GLL-19 (B), LoVo (C) and MDA-MB468 (D) cells. Control cells (○); cells exposed to bryostatin 1 (●). LAK cytotoxicity was evaluated at different effector:target ratios. Each point is the average of quadruplicate determinations. Standard errors never exceeded 10%.

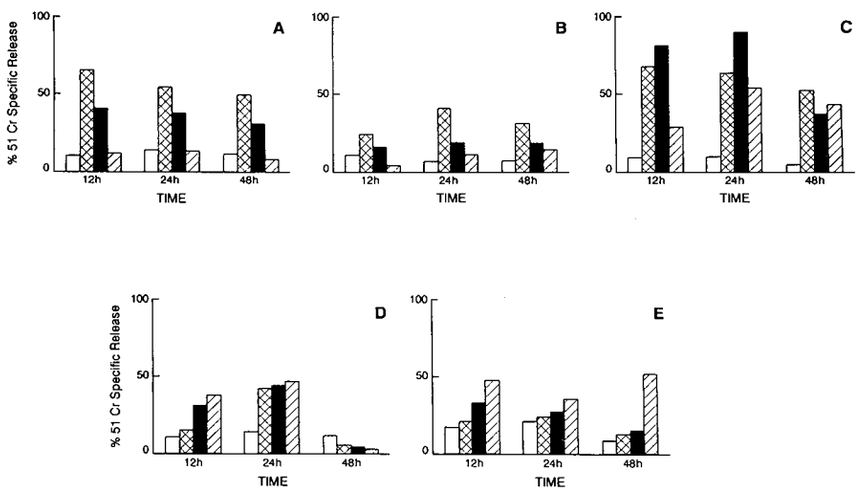

Figure 2. Dose-dependency and time-dependency of bryostatin 1 effects on A549 (A), GLL-19 (B), LoVo (C) and MDA-MB468 (D) cells. Control cells (□); 10 nM (▨), 1 nM (■) and 0.1 nM (▨) bryostatin 1. LAK cytotoxicity was evaluated at effector:target ratio = 20:1. Each point is the average of quadruplicate determinations. Standard errors never exceeded 10%.

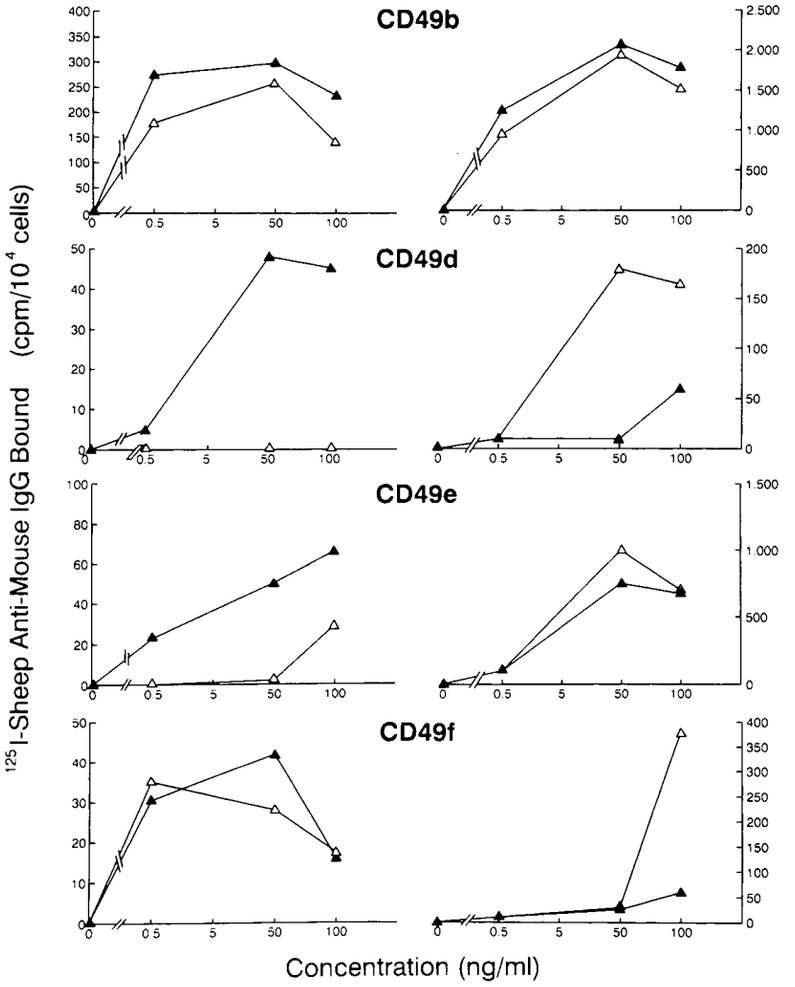

Figure 3. Bryostatin 1 effects on α^2 (CD49b), α^4 (CD49d), α^5 (CD49e) and α^6 (CD49f) expression on LoVo cells. Control cells (△); cells exposed to bryostatin 1 (▲). Left panel: LoVo cells; right panel: A549 cells. Each point is the average of quadruplicate determinations. Standard deviations were always less than 10%.

Conclusion

We have demonstrated that bryostatin 1 modulates important properties of human tumor cells such as integrin expression and sensitivity to cell-mediated cytotoxicity. Our results suggest that bryostatin 1 could be highly effective if administered in combination with immunostimulating agents, given its capacity to induce cytokine expression[13] and to sensitize human tumor cells to activated lymphocytes.

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