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Research Article

Factor(s) from nonmacrophage bone marrow stromal cells inhibit Lewis lung carcinoma and B16 melanoma growth in mice

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Abstract. Bone marrow stroma produces positive and negative growth regulators which constitute the hematopoietic microenvironment. As many tumors metastasize to the bones, these regulators may also influence tumor growth. Hematopoietic cytokines may indeed exert both positive and negative effect on tumor growth. We report that, when mixed with tumor cells, adherent bone marrow cells inhibit primary tumor growth and metastases formation in mice transplanted with Lewis lung carcinoma or B16 melanoma. Peritoneal macrophages or lymph node cells did not exert any influence. The tumor inhibition was apparently due to soluble factor(s) released by marrow stromal cells. In cocultures with B16 melanoma cells, adherent bone marrow cells exerted a significant antiproliferative effect which was increased by previous culture of the bone marrow cells with granulocyte-macrophage colony-stimulating factor but not with macrophage colony-stimulating factor. Neither neutralizing antibodies against tumor necrosis factor-α, transforming growth factor-β or interferon α/β nor addition of *Escherichia coli* lipopolysaccharide to generate inflammatory cytokines could affect the antiproliferative effect of bone marrow stromal cells. The bone marrow stroma factor(s) which inhibit tumor growth might, therefore, be a novel growth regulator.

Key words. Bone marrow stroma; negative growth regulator; antiproliferative effect; tumor growth; lung metastases.

The hematopoietic microenvironment influences the growth and differentiation of hematopoietic cells. Although not completely understood, this microenvironment is comprised of soluble regulatory factors, cellular elements and extracellular components. Soluble factors and cytokines which may exert both positive and negative effects on cell growth are mainly produced by adherent stromal cells of the bone marrow (BM) [1–3]. These positive and negative growth regulators may also affect hematopoietic tumors and carcinoma cells, which frequently metastasize to the marrow. Some in vitro studies suggest that BM stroma stimulates tumor growth and metastasis formation [4–6]. However, cytokines such as interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF) and α/β interferons, which are constitutively expressed in marrow stromal cells [7–9], have been reported to exert antitumor effects [10–13]. The question we asked was whether adherent BM stromal cells may influence tumor growth and metastasis formation in vivo. Lewis lung carcinoma or B16 melanoma were mixed with freshly obtained or cultured adherent BM cells and

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transplanted into C57BL/6 mice. The presence of adherent BM cells in the tumor inocula inhibited both primary tumor growth and metastasis formation possibly via the direct action of a soluble factor(s) which seems to be different from inflammatory cytokines and from cytokines with known antitumor action. The production of this factor(s) seems to be increased in adherent BM cells previously cultured in the presence of GM-CSF.

Materials and methods

Mice. Female, 2–3-month-old C57BL/6 were purchased from Charles River Italia, Como, Italy. The animals were kept under a 12-h light:dark cycle at 21 ± 1 °C with free access to food and water.

Reagents. Anti-transforming growth factor-β (a-TGFβ) and anti-tumor necrosis factor-α (a-TNFα) monoclonal antibodies were purchased from Genzyme, Cambridge, MA, USA and from Pharmingen, San Diego, CA, USA, respectively. Anti-interferon α/β (a-IFN α/β) policlonal antibody was purchased from Research Diagnostic, Flanders, NJ, USA. GM-CSF and macrophage colony-stimulating factor (M-CSF) were purchased from PeproTech, London, England. *Escherichia coli* lipopolysaccharide (LPS, O127:B8) was purchased from Sigma, St. Louis, MO, USA.

Adherent BM cell. BM cells were collected, suspended in culture medium constituted by α-minimum essential medium (MEM) with 5% horse serum (HS) and incubated overnight at a concentration of 3.5×10^6 cells/ml in tissue culture flasks (Costar, Cambridge, MA, USA). After incubation, nonadherent cells were separated by three vigorous rinsings with 10 ml of culture medium. Adherent cells were harvested after addition of EDTA 5 mM for 10 min at 4 °C. In certain experiments adherent BM cells were cultured for 1 week in α-MEM containing 12.5% foetal calf serum (FCS), 12.5% HS, 0.2 mM myoinositol, 0.02 mM folic acid, 0.1 mM mercaptoethanol, 1 μM hydrocortisone and 2 mM L-glutamine. Half of the culture medium was replaced twice a week. The cultures were then continued for a further week in the presence or absence of 100 U/ml of GM-CSF or M-CSF.

Peritoneal macrophages and lymph node cells. Peritoneal macrophages were collected by injecting 7 ml of α-MEM intraperitoneally. After washing the peritoneum, the medium was collected under the spleen by turning the mouse on the left side. Lymph node cells were obtained from the axillary, inguinal and peritoneal lymph nodes that were teased in α-MEM by a loose-fitting teflon pestle. Both macrophages and lymphocytes were washed 3 × in saline and adjusted at a concentration of 4×10^5 cells/100 μl.

Lewis lung carcinoma and B16 melanoma. LLC was maintained by serial passages in C57BL/6 mice as [14]. For the experiments, the primary tumor was excised from donor mice and teased by a loose-fitting teflon pestle, and the cells were dissociated to obtain a single-cell suspension in saline. After washing, the cells were counted, and 2×10^5 viable cells suspended in 200 μl of saline with or without 4×10^5 adherent BM cells were injected intramuscularly (i.m.) in the left hind leg. Control groups included animals inoculated with LLC cells

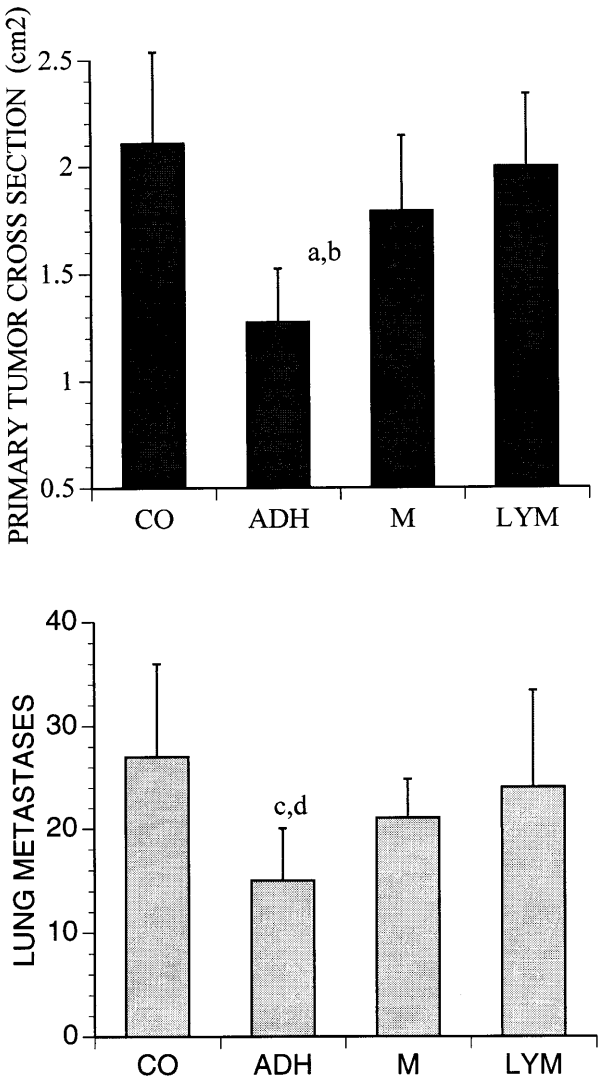

Figure 1. Effect of adherent BM cells on LLC growth in vivo. C57BL/6 mice were randomized and injected i.m. with LLC cells alone (CO) or mixed with adherent BM cells (ADH), peritoneal macrophages (M) or lymph node cells (LYM). Each experimental group consisted of 26 mice, and the values reported are the mean ± the standard deviation. a: $P < 0.002$ ADH vs. CO, LYM; b: $P < 0.02$ ADH vs. M; c: $P < 0.01$ ADH vs. CO, LYM; d: $P < 0.05$ ADH vs. M.

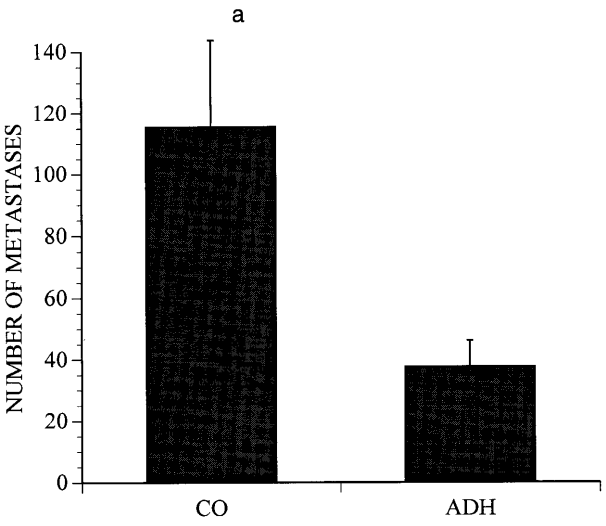

Figure 2. Effect of adherent BM cells on B16 melanoma lung metastases. Mice were injected i.v. with B16 melanoma cells alone (CO) or mixed with adherent BM cells (ADH). The columns represent the mean values of 12 mice per group ± the standard error. a: $P < 0.02$.

plus 4×10^5 peritoneal macrophages or lymph node cells. Primary tumor size and number of lung metastases were evaluated 20–22 days after tumor inoculation. Primary tumor size was evaluated by measuring the diameter of the leg at the tumor level with a caliper in two perpendicular directions. The tumor cross-section is considered an ellipse, and its size was calculated according to [15]:

$$\text{tumor cross section (cm}^2) = \Pi/4[(t1t2)-(c1c2)]$$

where t1 and t2 are the perpendicular axes of the tumorous leg and c1 and c2 are the perpendicular axes of the right control leg. Lung metastases were enumerated in the dissected lungs by a dissection microscope. B16 melanoma cells were cultured in IMDM, 10% foetal calf serum (FCS), tetracycline 10 μg/ml at 37 °C, 5% CO_2. After two washings in saline, cells were incubated with 0.05% trypsin, 0.02% EDTA for 1 min at 37 °C, then the trypsin and EDTA solution were discarded and the cells incubated for a further 10 min. The cells were then harvested in saline and washed 1 ×, counted and adjusted at a concentration of 2×10^5 viable cells in 100 μl. To establish experimental lung metastases, 10^5 B16 cells (50 μl) were added to 50 μl of saline with or without 5×10^5 adherent BM cells and inoculated intravenously (i.v.) through the retroorbital plexus. The number of established lung metastases was evaluated 20–21 days after tumor inoculation.

In vitro ^{3}H-thymidine incorporation. Adherent cell cultures were set up as described above on the bottom surface of transwell plates (3-μm pore size, Costar, Cambridge, MA, USA). After 2 weeks, in the presence or absence of growth factors during the 2nd week of culture, the transwells were washed to discard nonadherent cells, and 5×10^4 B16 viable melanoma cells were seeded on the upper mobile dish of the transwells and incubated together with the adherent BM cells in α-MEM, 5% FCS and 5% HS for 2 days. In this way the 3-μm pore size membrane constituting the physical support of B16 melanoma cells allowed any soluble substance to diffuse freely from the adherent cell layer to the tumor cells. When tested, a-TGFβ (20 μg/ml), a-TNFα (20 μg/ml), a-IFN α/β (100 U/ml) or lipopolysaccharide (LPS) (0.5 μg/ ml) were added just after the melanoma cells and incubated with adherent BM cells previously grown in the presence of GM-CSF for 2 days. The cultures were then pulsed with 1 μCi/well of ^{3}H-thymidine (Du Pont de Nemours, NEN products, Regensdorf, Switzerland) overnight. After washing with saline, the transwell mobile dishes bearing B16 melanoma cells were transferred to new plates where the cells were detached by incubation with 1 ml of 0.25% trypsin in saline for 20 min at 37 °C. The cell suspension obtained was then transferred in 10 ml of scintillation fluid and counted in a beta counter.

Statistics. Differences were evaluated for significance by analysis of variance (ANOVA).

Results

Effect of adherent BM cells on LLC and B16 melanoma growth in vivo. Adherent BM cells mixed with LLC cells inhibited primary tumor growth and significantly decreased the number of lung metastases (fig. 1). On the contrary, equal numbers of peritoneal macrophages and lymph node cells mixed with LLC cells did not influence tumor growth and metastasis formation. Actually, in these control groups, primary tumor size and number of metastases were very similar to those obtained from mice inoculated with tumor cells alone. In a different model, adherent BM cells added to B16 melanoma cells inoculated i.v. also decreased the number of established B16 lung metastases in a significant manner (fig. 2).

In vitro studies. To verify whether the effect observed in vivo was due to a direct action of soluble factor(s) released by adherent BM cells, we set up cocultures of adherent BM cells and B16 melanoma cells using the transwell system (see 'Materials and method'). Figure 3 shows that adherent BM cells produce soluble factor(s) which, by diffusing in the culture medium, inhibit ^{3}H-thymidine incorporation in B16 melanoma cells. When adherent BM cells were cultured for 2 weeks, the inhibition was stronger and further increased by addition of GM-CSF during the last week of culture. Interestingly,

M-CSF, a macrophage-specific growth factor, did not increase the inhibitory effect but rather decreased it. Consistently, peritoneal macrophages used as a control did not influence ^{3}H-thymidine incorporation in B16 melanoma cells (fig. 3).

In the attempt to identify the inhibitory factor, we evaluated whether antibodies against cytokines which have been reported to exert a direct antitumor effect could neutralize the inhibitory effect of adherent BM cells cultured in presence of GM-CSF. In addition, an experimental group was included in which LPS was added to induce the production of inflammatory cytokines in the culture (fig. 4). Neither the anticytokine antibodies nor LPS could significantly affect ^{3}H-thymidine incorporation in B16 melanoma cells (fig. 4). The putative stromal cell antitumor factor might therefore be an as yet unknown negative growth regulator.

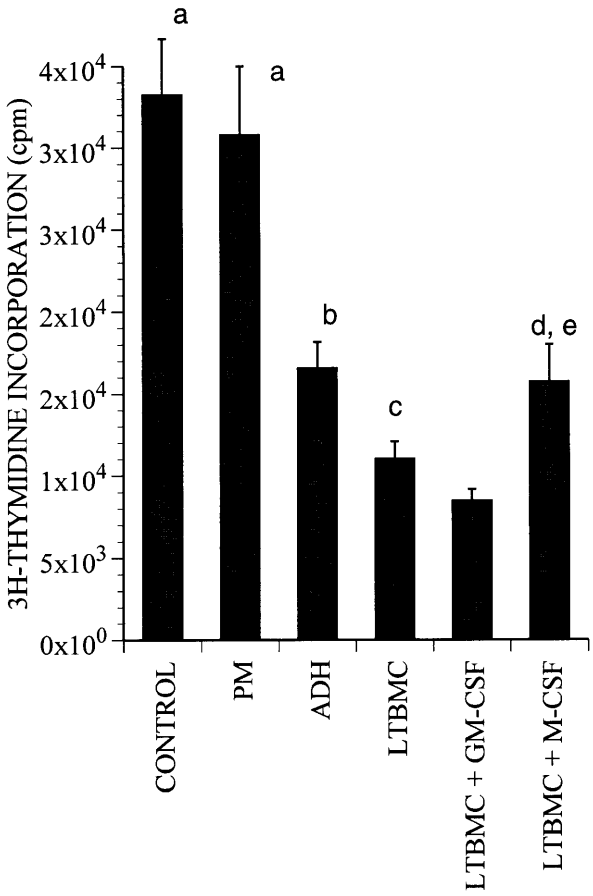

Figure 3. Inhibition of ^{3}H-thymidine incorporation in B16 melanoma cells by adherent BM cells. Freshly separated adherent BM cells (ADH) or after long-term culture (LTBMC) in the presence or absence of GM-CSF were incubated with B16 melanoma cells for 2 days before ^{3}H-thymidine pulsing. Control groups were B16 cells alone (CONTROL) and B16 cells incubated with peritoneal macrophages (PM). The values expressed in counts per minute (cpm) represent the mean from three experiments $\pm$ the standard deviation. a: $P < 0.001$, CONTROL and PM vs. all; b: $P < 0.01$, ADH vs. LTBMC, LTBMC + GM-CSF; c: $P < 0.02$, LTBMC vs. LTBMC + GM-CSF; d: $P < 0.05$, LTBMC + M-CSF vs. LTBMC; e: $P < 0.01$, LTBMC + M-CSF vs. LTBMC + GM-CSF.

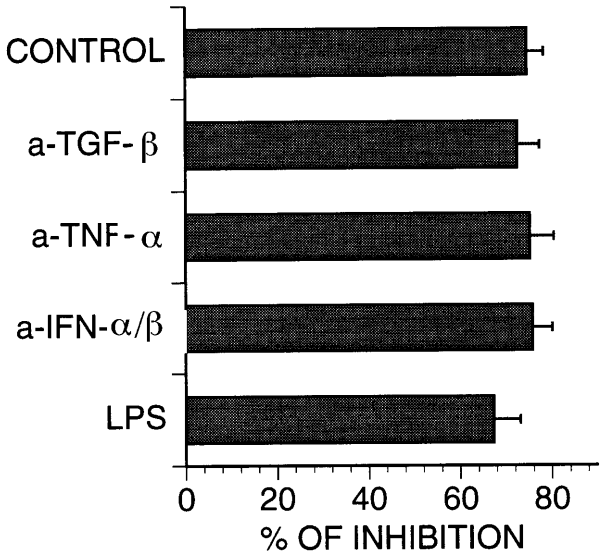

Figure 4. Anticytokines antibodies and/or LPS did not affect inhibition of ^{3}H-thymidine incorporation in B16 cells. Monoclonal or policlonal antibodies against the above-reported cytokines or LPS were added just after seeding B16 melanoma cells in transwell cultures of adherent BM cells previously grown in the presence of GM-CSF. Adherent BM cells + GM-CSF were the control. The values are expressed as percentage of inhibition of ^{3}H-thymidine incorporation in B16 melanoms cells and are the mean of three experiments $\pm$ the standard deviation.

Discussion

We demonstrated that adherent BM cells exert a significant inhibitory action on primary tumor growth and metastases formation in vivo. A mechanism which might explain this effect would be dependent on antigen-presenting cells such as dendritic cells or macrophages, which may yield effective immune responses against tumors in mice [16, 17]. However, peritoneal macrophages or lymph node cells did not exert any effect. In addition, dendritic cells are only a trace population in adherent BM cells [18]. Therefore, it seems unlikely that the antitumor effect is related to any activation of a specific immune response. Unseparated BM cells have also been reported to inhibit tumor growth by a mechanism involving cell-to-cell interaction [19]. We cannot exclude that this type of mechanism occurred in our in vivo experiments. However, adherent

BM cells but not peritoneal macrophages cocultured with B16 melanoma cells inhibited ^{3}H-thymidine incorporation in B16 melanoma cells, apparently by the secretion of soluble factor(s). It must be stressed that inhibition of ^{3}H-thymidine incorporation was parallaled by an evident inhibition of cell growth as evaluated by microscopic examination (not shown). This effect was increased by previous culture of adherent BM cells especially in presence of GM-CSF. Culture of adherent BM cells with M-CSF did not increase the inhibitory effect but rather decreased it. This suggested that the BM stroma cells which produce the antitumor factor are not macrophages but GM-CSF-sensitive stromal cells whose identification requires further studies.

In regard to the nature of this factor(s), antibodies against cytokines such as TNFα, IFNα/β and TGFβ did not neutralize the factor(s) action. Furthermore, addition of LPS, which is known to elicit the production of inflammatory cytokines, including interleukin-1, IL-6 and GM-CSF, did not exert any significant influence. On the other hand, cytokines such as IL-6 and TNFα have been also reported to enhance tumor metastases [20, 21]. We cannot exclude the involvement of known cytokines different from those taken in consideration. Nevertheless, present knowledge about the different cytokine effects allows speculation that we may have observed the effect of an as yet unknown growth-regulating substance. This seems a reasonable possibility in consideration of the complex and not yet fully understood function of BM stroma. On the other hand, factors with antitumor activity have been described in many different tissues [22]. Relevant to our finding, diffusible factors generated by a stromal cell line as well as in long-term BM cultures has been reported to preferentially inhibit leukemic cells rather than normal hematopoietic progenitor cells [23]. Further studies will be aimed at characterization of this factor(s) as well as at the elucidation of its pathophysiological importance and therapeutic potential.

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