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Interleukin-1 beta, transforming growth factor beta 1, prostaglandin E_2, and fibronectin levels in the conditioned mediums of bone marrow fibroblast cultures from lung and breast cancer patients

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Abstract We analyzed the ability of the bone marrow (BM) stromal cells to achieve confluence and their proliferative capacity in BM primary cultures from 30 untreated lung cancer patients (LCP), 27 breast cancer patients (BCP), and 30 normal controls (NC) when these confluent cells were induced to proliferate following four continuous subcultures. Moreover, we evaluated the production of interleukin-1 beta (IL-1β), transforming growth factor beta 1 (TGF-β1), fibronectin, and prostaglandin E_2 (PGE_2) by pure fibroblasts (fourth passage). A fibroblast colony-forming units (CFU-F) assay was used to investigate the proliferative and confluence capacity. Levels of IL-1β, TGF-β1, and fibronectin in conditioned mediums (CM) of fibroblast cultures were measured by enzyme-linked immunosorbent assay (ELISA) kit and PGE_2 by radioimmunoassay (RIA) kit. Confluence was achieved in the 60% of LCP and 78% of BCP primary cultures compared with 100% of NC, and only fibroblasts from seven LCP and six BCP cultures had the capacity to proliferate following four subcultures. Levels of IL-1β were below 10 pg/ml in both patient groups, while NC had a mean value of 5882.57±221.61 pg/ml. Levels of TGF-β1 in BCP were lower than NC values ($P<0.05$). LCP and BCP had significantly decreased levels of fibronectin when compared to NC values ($P<0.05$ and $P<0.01$, respectively). Levels of PGE_2 in LCP were higher compared to NC ($P<0.01$). In conclusion, BM fibroblasts from LCP and BCP presented a defective proliferative and confluence capacity, and this deficiency may be associated with the alteration of IL-1β, TGF-β1, fibronectin, and PGE_2 production.

Keywords Fibroblasts · Soluble factors · Cancer

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Introduction

The works of Friedenstein and Castro-Malaspina [3, 10] provided evidence that bone marrow (BM) contains, in addition to the hematopoietic progenitors, a population of spindle-shaped clonogenic fibroblast precursor cells or fibroblast colony-forming units (CFU-F). These plastic adherent cells, which were defined in vivo as quiescent resting cells, can continue the cell cycle and develop colonies after in vitro stimulation with selected batches of fetal bovine serum (FBS) [3]. In the past few years [1, 6, 25], these cells have attracted interest because of their ability to serve as a feeder layer for the growth of hematopoietic stem cells, their capacity for self-renewal, their multipotentiality for differentiation into at least seven types of cells (osteocytes, chondrocytes, adipocytes, tenocytes, myotubes, astrocytes, and hematopoietic-supporting stroma), and their possible use for both cell and gene therapy. These observations suggest that fibroblastic progenitors are an important component of the BM hematopoietic microenvironment and that the evaluation of these adherent cells in vitro may reflect the functional states of an in vivo microenvironment.

In previous studies, we demonstrated that CFU-F incidence was very low or that clusters were the only or the predominant type of clonal growth in BM primary cultures from the majority of untreated LCP and BCP [4, 5]. This reduction in the number of CFU-F may be

related to an increase in the levels of inhibitor factors and/or to a decrease in the levels of stimulant mediators such as interleukin-1 (IL-1), IL-17, platelet-derived growth factor (PDGF), transforming growth factor beta 1 (TGF-β1), epidermal growth factor, collagen, fibronectin, IL-2, interferon-α, and prostaglandin E_2 (PGE_2) [9, 13, 17, 20, 21, 27, 28]. For this reason, we evaluated the ability of BM stromal cells (majority fibroblasts) to achieve confluence in primary cultures and their proliferative capacity following four continuous subcultures in consecutive untreated lung cancer patients (LCP), breast cancer patients (BCP), and normal controls (NC). At the same time, we studied the spontaneous release of IL-1β, TGF-β1, fibronectin, and PGE_2 in conditioned mediums (CM) of pure fibroblast cultures.

Materials and methods

Patients

BM samples were obtained from 30 NC, 30 consecutive untreated patients with non-small lung epidermoid carcinoma (stage IIIA, IIIB, and IV), and 27 consecutive untreated patients with breast carcinoma (stage III and IV). We used a Union Internationale Contra la Cancrum (UICC) tumor node metastasis (TNM) classification. All LCP, BCP, and NC were age and sex matched. All the individuals gave consent to participate in these studies, which were performed in accordance with the principles of the Declaration of Helsinki. Patient BM aspirates were kindly provided by Dr. R.H. Bordenave and Dr. T. Angelillo Mackinlay from the Department of Oncology in the I. Iriarte and Británico Hospitals, respectively, Buenos Aires, Argentina. Healthy control BM aspirates were provided by Dr. E.O. Bullorsky from the Department of Hematology and Bone Marrow Transplantation in the Británico Hospital, Buenos Aires, Argentina. NC were healthy donors for allogeneic BM transplantation.

This present investigation was approved by the British and I. Iriarte Hospital Ethical Committees.

BM micrometastases

BM infiltration with neoplastic cells was detected by immunocytochemistry staining (Universal Dako LSAB System, biotin-streptavidin-peroxidase, DAKO Corporation, Carpinteria, Calif., USA), and an analysis of cell morphology was done by the Pappenheim technique. BM samples were stained with antibodies to epithelial membrane antigen (EMA, DAKO; for location, see above), to cytokeratin AE1-AE3 (DAKO), to cytokeratin 7 (CK7, DAKO), and to cytokeratin 20 (CK20, DAKO). Patients were considered positive for micrometastasis only if cells expressed EMA (breast cancer), cytokeratin AE1-AE3 (lung and breast cancer), CK7 (lung cancer), and/or CK20 (lung cancer), and if these were morphologically malignant.

Collection and preparation of BM cells

BM samples were collected under local anesthesia from the posterior iliac crest into heparinized saline without preservatives (25 U/ml, Gibco, Rockville, Md., USA). Aspirates were diluted 1/2 with phosphate-buffered saline (PBS, pH=7.5) and were layered on Histopaque (density=1,075g/cm^3, Sigma Chemical Company, St. Louis, Mo., USA). After being centrifuged for 25 min at 1500 rpm, mononuclear cells were harvested from the interface, washed twice in PBS, and resuspended in α-medium (Gibco; for location, see above) containing 100 IU/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 25 μg/ml of amphotericin B (Gibco). The cellular suspension was counted with 3% acetic acid solution, and cell viability was determined by trypan blue dye exclusion.

Confluent primary culture formation

A total of $5{\times}10^6$ viable light density mononuclear cells were placed in 25-cm^2 tissue cultured flasks (Corning, N.Y., USA), which contained 10 ml of supplemented previously described α-medium and 20% heat-inactive FBS (catalog number 16,000–044, Gibco). This supplemented medium is known to be selective for fibroblast progenitor proliferation. The cells were incubated in a 37°C, 5% CO_2 humidified environment for 7 days. After this period, the nonadherent cells were removed and the medium was renewed. The primary cultures were returned to incubation for further days until confluence. From the initiation of the experiment until day 60, or until the cells reached confluence, the medium was changed every 7 days. At the end, the medium was discharged and the confluent adherent cells were washed twice with PBS and trypsinized with a solution of trypsin-ethylenediaminetetraacetate (EDTA) (0.05%–0.02% in PBS, respectively, Gibco). Finally, trypsin-sensitive adherent cells were further induced to proliferate following four continuous subcultures. The adherent cells were subcultured only after confluence in each case. The number of days the adherent cells took to achieve confluence in primary cultures was studied, as well as the number of patients with fibroblastic proliferative capacity when cells from confluent primary cultures were further induced to proliferate following four subcultures.

The fibroblastic nature of the adherent cells that composed the cultures was demonstrated by immunofluorescent staining with monoclonal antibody against human fibronectin (gift from Dr. A. Kornbliht, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina) and against human β subunit of prolyl-4-hydroxylase (DAKO Corporation, Carpinteria, Calif., USA). In addition, we used cytochemical analyses with alkaline phosphatase stain. Cell samples were fixed with 50% methanol.

Preparation of CM from pure fibroblasts

BM fibroblasts isolated after four continuous subcultures were adjusted to $5{\times}10^4$ viable cells/ml in fresh α-medium, which contained 20% FBS and 1% antibiotic-antimycotic agent. The fibroblasts were incubated for 96 h at 37°C, 5% CO_2 in plastic tissue culture plates (Falcon 6 well plate, PGC Scientifics Corporation, Frederick, Md., USA). The CM were obtained by centrifugation at 1000 rpm for 10 min and frozen at –20°C before use for IL-1β and fibronectin assays and at –70°C for PGE_2 and TGF-β1.

Determination of IL-1β and PGE_2 in the CM of pure fibroblast cultures

IL-1β was measured by an enzyme-linked immunosorbent assay (ELISA) kit, developed and given to us by Dr. E. Eugui, California, and the PGE_2 was quantified by a radioimmunoassay (RIA) kit (DuPont). For both soluble factors, the CM were diluted 1:5. IL-1β assay detects levels between 10 and 7,400 pg/ml and the PGE_2 assay between 2.5 and 250 pg/ml.

Determination of fibronectin in the CM of pure fibroblast cultures

Fibronectin was determined by an ELISA of our own, according to the technique described by Gómez-Lechon [11] with modification: 100 ng/well of human plasma fibronectin (Gibco) were fixed in a Falcon 96 well microplate (PGC Scientifics Corporation, Frederick, Md., USA) and incubated overnight at 4°C. Simultaneously, samples and standards to perform a curve (0–5 μg/ml)

were incubated into glass tubes overnight at 4°C, in a 1:1 ratio, with a rabbit polyclonal antibody anti-human fibronectin (Gibco), diluted 1:25,000 in Tris-buffered saline (TBS).

The day after, the microplaque was washed five times with TBS plus 0.2% Tween 20 (TBS-T), and then the remaining sites were blocked with milk (100 µl/well of 200 mg/ml of fat milk in TBS-T) for 30 min at room temperature after the adhesion of protein in each well.

After the microplaque was washed five times with TBS-T, samples and standards binding to the antibody anti-human fibronectin were added to it (100 µl/well) for 60 min at room temperature. Then it was washed again five times with TBS-T and after that, we added a second antibody, goat anti-rabbit IgG-biotin conjugated (Gibco) in a 1:1000 dilution. After incubating for 60 min at room temperature, other series of washes were done. Next, the microplate was incubated with streptavidin-alkaline phosphatase conjugate (1:4000, Gibco) for 60 min at room temperature. Then the plate was washed again five times, and the enzyme was revealed by the addition of a mixture of substrate-chromogen solution (p-nitrophenyl phosphate, Sigma Chemical Company, St. Louis, Mo. USA, 1 mg/ml in diethanolamine buffer 1 M, pH=9.8).

The yellow color of the p-nitrophenyl phosphate was measured at an optical density of 405 nm in a photocolorimeter (Metrolab, Cambridge Technology Inc., Cambridge, Mass., USA) and the enzymatic reaction was stopped with 100 µl of NaOH 1 M at 0.8 optical density of the 0 ng/ml standard sample. For each assay, we employed a curve linealized with a logit-log system to obtain the values of concentration of fibronectin, and each curve was designed with the values of optical density vs the logarithm of nanogram of soluble fibronectin. The lowest concentration detectable was 5 ng/ml and the intra-assay and interassay variabilities were 5% and 6%, respectively.

Determination of TGF-β1 in the CM of pure fibroblast cultures

TGF-β1 was measured with an ELISA kit (R&D Systems, Minneapolis, Minn., USA). This assay detects levels between 31.2 and 2000 pg/ml. Simultaneously, we evaluated the levels of IL-1β, TGF-β1, fibronectin, and PGE_2 in samples of supplemented α-medium after 7 days of incubation and observed that the concentration for all the soluble factors was below the minimum detectable doses. All samples and standards were carried out in duplicate.

Statistics

Statistical analysis was evaluated by parametric and nonparametric tests depending on the data studied (P<0.05).

Results

BM micrometastases

Using the Pappenheim and immunocytochemistry techniques, morphological evidence of BM infiltration with neoplastic cells was not observed in cancer patients.

Evaluation of proliferative and confluence capacity of the adherent cells in BM primary cultures and four continuous subcultures

Figure 1 shows that only 6 of 30 (20%) BM cultures from LCP and 3 of 27 (11%) BM cultures from BCP had adherent cells, which were able to achieve confluence in primary cultures within the normal range (13–27 days).

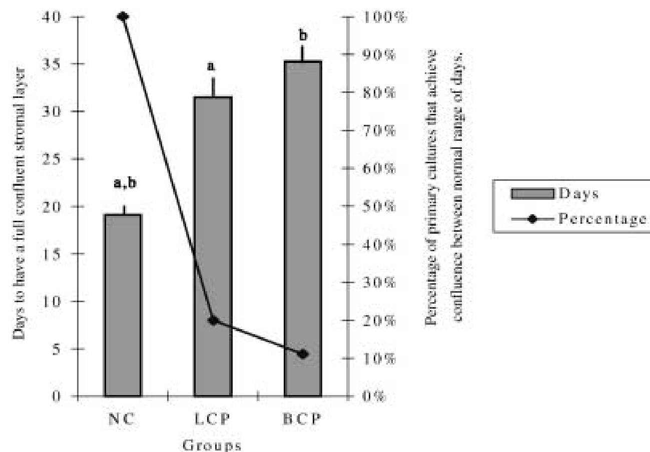

Fig. 1 Evaluation of confluence capacity of stromal adherent cells in primary cultures. Values are expressed as $\overline{X}$ of duplicated individual values. This mean was done with the patients who showed confluent adherent cells during the 60 days from the beginning of the incubation. Also, 40% of LCP and 22% of BCP did not reach confluence at all. Statistical significance: a, b=P<0.001. *LCP* lung cancer patients, *BCP* breast cancer patients, *NC* normal controls

Furthermore, in the other 24 cultures from LCP and BCP, we observed that there was lack of confluence or that more days were needed for the stromal layers to obtain confluence. The values $(\overline{X} \pm ES)$ in days were: LCP=31.50±2.16 days, BCP=35.23±2.46, and NC=19.07±0.75 days (P<0.001 for both groups, nonparametric Dunn's multiple comparisons test) (Fig. 1).

On the other hand, when adherent cells from confluent primary cultures of LCP (18/30) and BCP (21/27) were further induced to proliferate following four continuous subcultures, we observed that the growth had diminished or stopped in 11 of LCP and 15 of BCP. In contrast, all NC cultures presented full confluent stromal layers in primary cultures and had the capacity to proliferate following four continuous subcultures.

In terms of lineage markers, up to 100% of the nonhematopoietic adherent cells from patients and NC primary cultures expressed three fibroblast markers (fibronectin, prolyl-4-hydroxylase, and alkaline phosphatase). Moreover, the majority of fibroblast-like cells in confluent primary cultures presented a fusiform-shape in all groups.

However, the number of trypsin-EDTA-sensitive (0.05–0.02%) adherent cells in the confluent primary culture flask decreased in the BM cultures of LCP and BCP compared to the values obtained in NC (P<0.05 for both groups, parametric Dunnet multiple comparisons test, Fig. 2). It is well known that only BM adherent cells that have a fibroblastic nature are detached after this treatment [3]. But so far, we do not know if these fusiform-shaped adherent cells from LCP and BCP confluent primary cultures are bigger than NC cells. We need further evaluation to confirm this hypothesis.

Regarding the number of trypsin-EDTA-sensitive (0.05–0.02%) fibroblast-like cells in the fourth confluent subculture, the results showed that LCP and BCP presented no difference compared to NC values (para-

Table 1 Determination of the levels of IL-1β, PGE_2, TGF-β1, and fibronectin in the CM of pure fibroblast cultures. Values are expressed as $(\overline{X} \pm ES)$. *LCP* lung cancer patients, *BCP* breast cancer patients, *NC* normal controls

Groups	IL-1β (pg/ml)	PGE_2 (pg/ml)	TGF-β1 (pg/ml)	Fibronectin (μg/ml)
LCP (*n*=7)	<10	387.50±92.47[a]	621.72±103.26	9.37±1.30[b]
BCP (*n*=6)	<10	102.57±5.52	267.16±38.67[b]	5.54±1.97[a]
NC (*n*=7)	5,882.57±221.61	91.42±1.88[a]	768.28±163.82[b]	14.48±1.11[a, b]

[a] *P*=<0.01 [b] *P*=<0.05

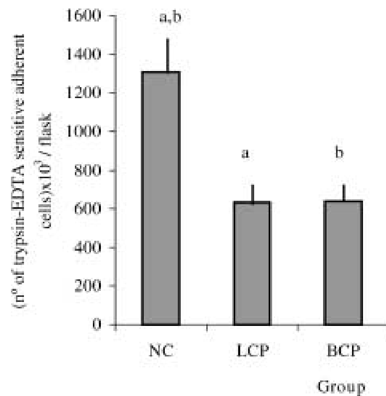

Fig. 2 Evaluation of the number of trypsin-EDTA sensitive BM adherent cells in confluent primary cultures. Values are expressed as $(\overline{X} \pm ES)$. Statistical significance: a, b=*P*<0.05

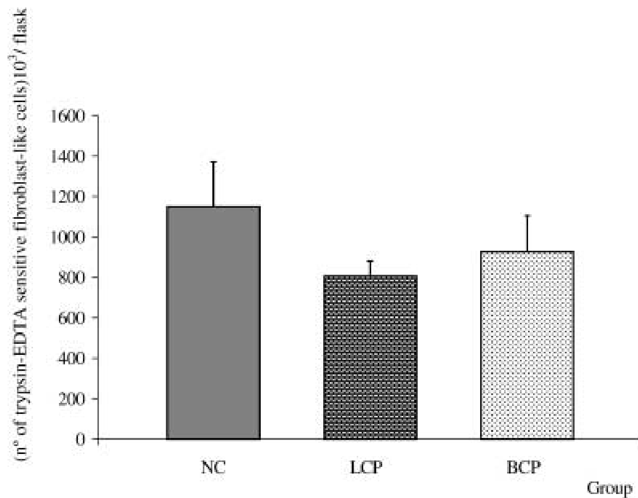

Fig. 3 Evaluation of the number of trypsin-EDTA-sensitive fibroblast-like cells in confluent fourth subculture. Values are expressed as $(\overline{X} \pm ES)$. Statistical significance: no differences

metric Dunnet multiple comparisons test, Fig. 3). Nevertheless, when these pure fibroblasts (fourth subculture) were incubated for 96 h in culture plates for the harvesting of the CM, only six of seven LCP cultures and three of six BCP cultures were able to achieve confluence.

Determination of the levels of IL-1β, TGF-β1, fibronectin, and PGE_2 in the CM of pure fibroblast cultures

The levels of IL-1β spontaneously released in the CM of fibroblast cultures from LCP and BCP were below detectable amounts (<10 pg/ml), while NC presented values of $(\overline{X} \pm ES) = 5,882.57 \pm 221.61 \text{pg/ml}$ (Table 1). This table also shows that the CM of fibroblast cultures from BCP had lower values of TGF-β1 compared to NC values (*P*<0.05, nonparametric Dunn's multiple comparisons test). However, the CM of LCP presented levels of TGF-β1 within the NC mean value.

In addition, the levels of fibronectin in the CM of fibroblast cultures from LCP and BCP had significantly decreased compared to those values seen in NC, with a *P*<0.05 and *P*<0.01, respectively (parametric Student-Newman-Keuls multiple comparisons test) (Table 1).

Finally, the levels of PGE_2 in the CM of fibroblast cultures from LCP increased significantly compared to the mean value obtained in NC cultures (*P*<0.01, nonparametric Dunn's multiple comparisons test) (Table 1). In contrast, no difference in the levels of this soluble factor was observed when BCP and NC groups were compared.

Discussion

BM stromal cells produce multiple factors (extracellular matrix and cytokines), which are not only capable of controlling the self-renewal, proliferation, and differentiation of hemopoietic stem/progenitor cells, but are also regulated by them [8, 22, 24, 27]. Functional alterations of BM microenvironment may be involved in the manifestation of some malignant disorders [7, 14].

The results presented in this paper showed that a high percentage of BM primary cultures from untreated LCP and BCP had phenotypic abnormalities such as diminished ability of the adherent stromal cells to achieve confluence or a lack of confluence. These observations are likely to reflect derangement in the composition and/or in the proliferative capacity of the stromal population, mainly fibroblastic progenitors. In previous studies, we found that CFU-F incidence was very low in primary BM cultures from LCP and BCP [4, 5]. In addition, when adherent cells from confluent primary BM cultures from LCP and BCP (majority fibroblasts) were further induced to proliferate following four continuous subcultures, a diminished or no further growth was observed in

23 of LCP and 21 of BCP. This defective proliferative potential of the fibroblasts in LCP and BCP might explain the lower ability of the stromal components to achieve confluence in primary cultures and in the other four subcultures.

The regulation of fibroblast proliferation is tremendously complex. The cellular shape seems to be one of the primary factors that regulate the mitogenic responses of fibroblasts to certain mitogenic agents [12]. This becomes evident when one considers cells (such as fibroblasts) in which the proliferative rate is anchorage dependent. When normal BM fibroblasts are plated on plastic, they will spread on the surface and proliferate actively in response to serum factors, until they reach confluence [12].

In our case, we observed that the primary BM cultures, where the adherent cells could not achieve confluence during the 60 days after the initiation of the incubation, presented cells with a round shape and vacuoles (data not shown). It is well known that in this supplemented medium the only normal progenitor that is able to proliferate is the fibroblast. As other authors have described, the cells that remained rounded no longer presented division in response to serum factors [12]. On the other hand, as mentioned before, fibroblast growth can be altered by many soluble factors [20, 27].

In the present study, we found that pure fibroblasts isolated after four continuous subcultures from BM of LCP and BCP released very low levels of IL-1β (<10 pg/ml) compared with NC. In contrast, pure fibroblast cultures from LCP released high levels of PGE_2 compared with NC values. This result sustains the theory that defective proliferative potential of the progeny of CFU-F can be caused by an increase in the production of PGE_2. These findings are in agreement with the report of other authors who observed that PGE_2 is an inhibitor of fibroblast proliferation in serum-supplemented cultures [15, 19]. This culture suppression may be related with an increase in the intracellular cAMP levels [9, 18].

Besides, LCP and BCP had low levels of fibronectin in the CM of pure fibroblast cultures. Therefore, fibroblast proliferation may be associated with a lower production of this component of the extracellular matrix. In vitro, the extracellular matrix components can stimulate fibroblast growth directly or can induce the release of cytokines that play an important role during fibroblast proliferation [2, 26]. Moreover, some cytokines such as PDGF and TGF-β1 stimulate the production of fibronectin [16, 23]. Our study also showed that CM of pure fibroblast cultures from BM of BCP presented low levels of TGF-β1 compared to NC values. These findings may be related with the decreased levels of fibronectin observed in the CM of fibroblast cultures from BM of BCP.

Analyzing all the results, we arrived at the conclusion that BM fibroblasts from LCP and BCP had a defective proliferative and confluence capacity not only in the primary cultures but also during the following four subcultures. Moreover, the pure fibroblasts from both groups of patients had an alteration of IL-1β, TGF-β1, fibronectin, and PGE_2 production that may play an important role in the regulation of the proliferative and confluence capacity of these stromal cells. Finally, although it seems clear that functional abnormalities in the BM fibroblasts exist in LCP and BCP, it is not known whether these modifications are an inherent defect in fibroblasts of patients susceptible to lung and breast carcinoma or an acquired defect related to the tumor itself. Consequently, we need further evaluation.

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