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Alteration on the Expression of IL-1, PDGF, TGF-β, EGF, and FGF Receptors and c-Fos and c-Myc Proteins in Bone Marrow Mesenchymal Stroma Cells from Advanced Untreated Lung and Breast Cancer Patients

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ABSTRACT

Previously, we reported a deficient cloning capacity of the bone marrow (BM) mesenchymal stem cells to give colony-forming unit fibroblast (CFU-F) and an inefficient confluence capacity of BM stromal cells in advanced untreated lung cancer patients (LCP) and breast cancer patients (BCP). Moreover, a decreased level of bFGF at day 7 in the conditioned media from BM CFU-F cultures was found in both cancer groups when compared to the normal range. The current study was specially undertaken, to evaluate the percentage of subconfluent fibroblasts expressing receptors (R) of interleukin-1 (IL-1), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF-β), epidermal growth factor (EGF), and the proteins c-Fos and c-Myc in BM primary cultures from untreated LCP and BCP. An immunocytochemical study on subconfluent BM fibroblast cultures from 13 healthy patients, 16 LCP, and 8 BCP was performed, using as primary antibodies, anti-type I of IL-1 R (IL-1R-1), anti-α, β chains of PDGF R (PDGFR-α, PDGFR-β), anti-type I of FGF R (FGFR-I), anti-type I, II, and III of TGF-β R (TGF-βR-I, TGF-βR-II, and TGF-βR-III), anti-EGF R, anti-c-Fos, and anti-c-Myc. A diminished percentage of subconfluent fibroblasts expressing PDGFR-α, TGFβR-I, II, III, EGFR, and FGFR-I was found in LCP and BCP compared to healthy patients. A diminished percentage of subconfluent fibroblasts expressing c-Fos and c-Myc was found in patients when compared to healthy patients. The alterations we describe could help to explain the deficiency regarding the proliferative and confluence capacity of BM stroma cells in cancer patients.

INTRODUCTION

THE BONE MARROW (BM) hematopoietic microenvironment consists of stromal cells (macrophages, fibroblasts, adipocytes, and endothelial cells), accessory cells, extracellular matrix components, and soluble factors (stimulant and inhibitor). Marrow stromal fibroblasts, of particular concern in this study, are plastic-adherent and nonphagocytic cells growing on a major proportion on mesenchymal stromal cell (MSC) cultures

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(1,2). In cultures generated from a single-cell suspension of marrow, MSCs grow in colonies each derived from a single precursor cell called a colony-forming unit fibroblast (CFU-F) or mesenchymal stem cell (1). A proportion of CFU-F demonstrates extensive proliferative potential both in vivo and in vitro. In vitro, this extensive proliferative potential of a subset of CFU-F has been shown to be associated with a capacity for extensive self-renewal (3). On transplantation in vivo, the progeny of a proportion of CFU-F have been shown to be capable of proliferating and differentiating into all of the stromal cell lines, with the exception of macrophages, necessary for the formation of bone and reconstitution of hematopoietic inductive microenvironment (4–6).

Table 1. Polyclonal and Monoclonal Antibodies Used to Evaluate the Percentage of BM Subconfluent Fibroblasts Expressing Receptors of PDGF, TGF-β, EGF, FGF, and IL-1

Antibodies (Ab)	*Dilutions*	*Isotypes*
Rabbit Pc Ab anti-human (h) type I TGF-β R (Santa Cruz) (200 μg/ml)	1:67	IgG
Rabbit Pc Ab anti-h type II TGF-β R (Santa Cruz) (200 μg/ml)	1:67	IgG
Goat Pc Ab anti-h type III TGF-β R (Santa Cruz) (200 μg/ml)	1:67	IgG
Rabbit Pc Ab anti-h type I IL-1 R (Santa Cruz) (200 μg/ml)	1:67	IgG
Mouse mAb anti-h EGF R (Oncogene) (100 μg/ml)	1:33	IgG_{2a}
Mouse mAb anti-h type I FGF R (Oncogene RP) (100 μg/ml)	1:10	IgG_1
Rabbit Pc Ab anti-h β chain PDGF R (Santa Cruz)	1:10	IgG_1
Rabbit Pc Ab anti-h α chain PDGF R (Santa Cruz) (200 μg/ml)	1:67	IgG
Mouse mAb anti-h macrophage CD68 (Dako) (460 μg/ml)	1:150	IgG_1
Mouse mAb anti-h fibroblast (prolyl-4-hydroxylase) (Dako) (150 μg/ml)	1:50	IgG_1
Negative controls		
Mouse 1st Ab isotype control (IgM and IgG) (Zymed) (0.5 μg/ml)	All adjusted to the same mouse isotype IgG_1, IgG_2 or total IgG concentration previously described	
Mouse IgG_1 mAb (anti-aspergillus niger glucose oxidase) (Dako) (100 μg/ml)		
Mouse IgG_{2a} mAb (anti-aspergillus niger glucose oxidase) (Dako) (200 μg/ml)		
Goat immunoglobulin negative control (Bethyl) (500 μg/ml)		
Rabbit immunoglobulin negative control (Dako) (1,500 μg/ml)		

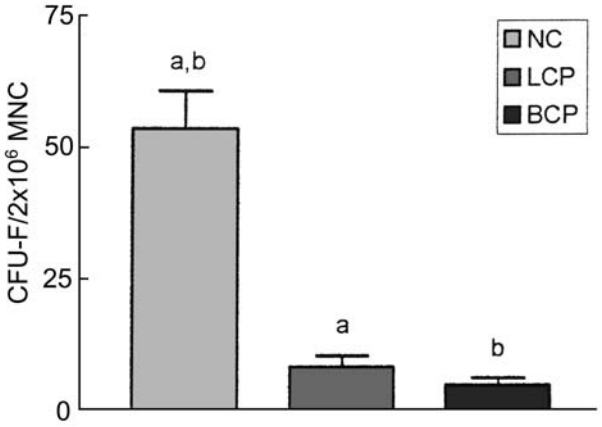

FIG. 1. Number of CFU-F per 2×10^6 MNC. Values are expressed as X ± SE. Statistical significance: a, b = $p < 0.001$.

For CFU-F formation and proliferation, the interrelation between the mesenchymal stem cell, the committed progenitors, and the hematopoietic progenitors is necessary, as well as the extracellular matrix (ECM) components and certain soluble factors released by them. Several soluble factors enhance the growth of CFU-F and fibroblasts in vitro, platelet-derived growth factor (PDGF)-AA, AB, BB, EGF, transforming growth factor-β (TGF-β) (dose- and high cell density-dependent manner), basic fibroblast growth factor (bFGF), interleukin-1 (IL-1) (in an indirect way), granulocyte-macrophage colony-stimulating factor (GM-CSF) (in dose-dependent manner), and IL-17 (7–9). Some factors inhibit in an indirect or direct way, like IL-2, interferon-α (IFN-α), and IFN-γ, IL-4, tumor necrosis factor-α (TNF-α) (dose-dependent manner), PGE2, and IL-10 (7).

Growth factors are involved in the regulation of growth, replication, and differentiation of BM stromal fibroblasts depending upon their interaction with their specific high-affinity receptor located on each cell membrane (10). It is well known that growth factors elicit their biological effects only after binding to their specific plasma membrane receptor, and it is only after this event that the signal transduction cascade can follow and the cell cycle progress (11). One of the mechanisms by which a stimulant factor of CFU-F formation and proliferation acts is through the stimulation of the expression of the mRNA of the proto-oncogenes c-*fos* and c-*myc* (12). This increase in the expression of mRNA occurs after the union of the blastogenic factor to the specific receptor on the membrane of the cell and is related to the activation of the quiescent MSC to go on into the cell cycle from G_0 to G_1. This last event happens because proto-oncogenes encode for nuclear proteins that are involved in the regulation of the transcription of other important genes implicated in the progression of the cellular cycle (13,14).

In stromal fibroblasts, c-Myc and c-Fos expression is continuously and tightly regulated by mitogen availability. Following mitogen stimulation, quiescent stromal fibroblasts show rapid induction of c-Myc and c-Fos whereas the neutralization or inactivation of the mitogen causes c-Myc and c-Fos mRNA to decrease and growth to become arrested in G_1 (14–16).

In previous studies, we found in our laboratory that most BM primary cultures from untreated lung cancer patients (LCP) and breast cancer patients (BCP) had a decreased number of CFU-F. Also, we observed a deficient confluence capacity of the MSC with a fibroblastic nature (prolyl-4-hydroxylase$^+$) from the BM primary cultures of untreated advanced LCP and BCP, a deficiency that was also present in the future subcultures (17–19). Moreover, the conditioned media from these BM CFU-F cultures (7 and 14 days) of both cancer groups suppressed or reduced the cloning efficiency of the mesenchymal stem cells to produce CFU-F from normal BM (20). And only a significant decrease and increase of the levels of basic FGF and GM-CSF, respectively, were found in the conditioned media of 7 days from CFU-F cultures of LCP and BCP compared to normal values. No other changes regarding the other classical stimulant and inhibiting factors were found (21). These data indicated to us the importance of studying the classical growth factor receptors and the expression of c-Fos and c-Myc proteins in BM subconfluent fibroblast cultures from untreated, advanced LCP and BCP. The purpose of this study was to evaluate some of the factors involved in the deficient proliferative and confluence capacity previously described in our cultures.

MATERIALS AND METHODS

Patients

BM samples were obtained from 13 healthy patients, 16 consecutive untreated patients with non-small cell lung epidermoid carcinoma (stage III A, III B, and IV, LCP), and 8 consecutive untreated patients with breast carcinoma (stage III and IV, BCP). We used a UICC TNM classification. All LCP, BCP, and healthy patients were age and sex matched. All individuals gave consent to participate in these studies, which were performed in accordance with the principles of the Declaration of Helsinki. Healthy patients were healthy donors from allogeneic BM transplantation. This present investigation was approved by the British and I. Iriarte Hospital Ethical Committees.

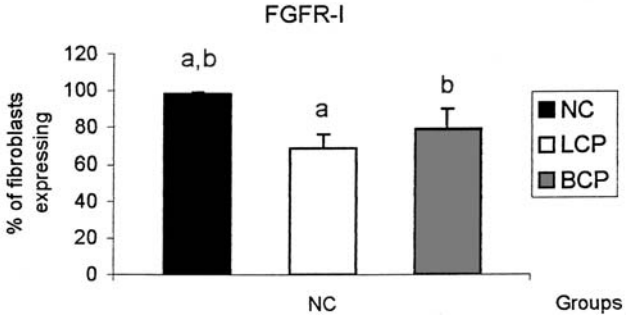

FIG. 2. Percentage of subconfluent fibroblasts expressing FGFR-I. Values are expressed as X ± SE. Statistical significance: a, b = $p < 0.03$.

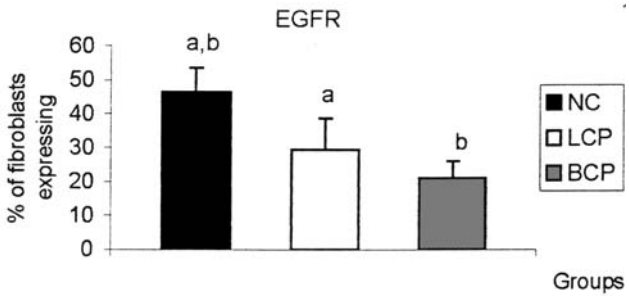

FIG. 3. Percentage of subconfluent fibroblasts expressing EGFR. Values are expressed as X ± SE. Statistical significance: a = $p < 0.03$, b = $p < 0.007$.

BM micrometastases

BM infiltration with neoplastic cells was detected by immunocytochemistry staining [streptavidin-peroxidase conjugate/diaminobenzidine (DAB) chromogen; Universal Dako LSAB System], and an analysis of cell morphology was performed using the Pappenheim technique. BM samples were stained with monoclonal antibodies (mAbs) to epithelial membrane antigen (EMA; Dako), cytokeratin AE1-AE3 (Dako), cytokeratin 7 (CK7; Dako), and 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 CK20 (lung cancer) and if the cells were morphologically malignant.

Collection and preparation of BM cells

BM samples were collected under local anesthesia from posterior iliac crest into heparinized saline without preservatives (25 units/ml, Gibco). Aspirates were diluted half with phosphate-buffered saline (PBS, pH 7.5) and were layered on Histopaque (density 1.075 gr/cm^3, Sigma). After being centrifuged for 25 min at 1,500 rpm, BM light-density mononuclear cells were harvested from the interface, washed twice in PBS, and resuspended in α-medium (Gibco) containing 2 mM L-glutamine (Gibco), 100 IU/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 25 μg/ml of amphotericin B

(Gibco) (supplemented α-medium). The cellular suspension was counted with 3% acetic acid solution and cell viability was determined by Trypan Blue dye exclusion.

CFU-F assay

A CFU-F culture was developed following the technique described by Castro Malaspina (22).

Evaluation of the percentage of subconfluent (85–90%) fibroblasts expressing receptors of PDGF, TGF-β, EGF, FGF, and IL-1 in BM primary cultures from LCP, BCP, and healthy patients

An immunocytochemical method (streptavidin-peroxidase System/DAB chromogen) using polyclonal and mAbs against human IL-1R-I (Santa Cruz Co.), PDGFR-α and β (Oncogene), FGFR-I (Oncogen), TGF-βR-I, II, and III (Santa Cruz Co.), and EGFR (Oncogen) was done. Negative controls were performed without the first Ab and with mAbs with the same isotype of the primary Ab (see Table 1). A total of 5×10^5 viable BM light-density mononuclear cells were placed in a plastic chamber slide (8-wells/slide; Labtec de Nunc Co), which contained 500 μl of supplemented α-medium (Gibco) and 20% heat-inactivated fetal bovine serum (FBS; Gibco cat. # 16,000-044). This medium is known to be selective for fibroblast progenitors proliferation (22). 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 renewed. The primary cultures were returned to incubation for further days until subconfluence. The medium was changed every 7 days. At the end, the medium was discharged and the subconfluent adherent cells were washed twice with PBS. The adherent cells were placed at −20°C until used. The samples were thawed at room temperature before use and fixed with 100% methanol. The immunocytochemical technique was then followed as indicated by the manufacturer. Staining for PDGFR-β was technically unsatisfactory and the low signal could not be quantified in the majority of the samples.

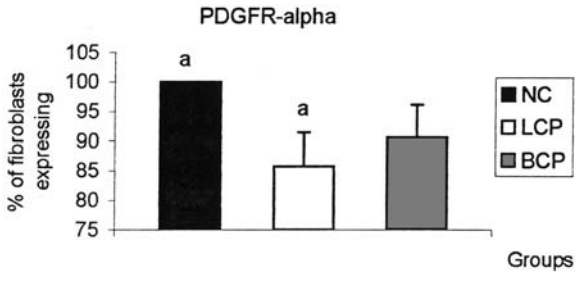

FIG. 4. Percentage of subconfluent fibroblasts expressing PDGFR-α. Values are expressed as X ± SE. Statistical significance: a = $p < 0.009$.

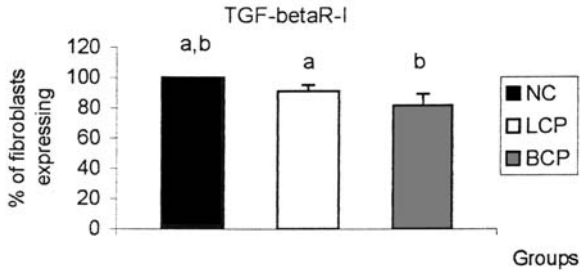

FIG. 5. Percentage of subconfluent fibroblasts expressing TGF-βR-I. Values are expressed as X ± SE. Statistical significance: a = $p < 0.03$, b = $p < 0.02$.

The fibroblastic nature of the adherent MSC of the cultures was demonstrated by immunocytochemical technique (streptavidin-peroxidase System/DAB chromogen) with mAbs against human fibronectin (Dako) and against the human β-subunit of prolyl-4-hydroxylase (Dako). In addition, a cytochemical analysis with alkaline phosphatase stain was developed. A mAb anti-CD68, which develops a low stain over fibroblasts and a high stain for macrophages, was also done. The spindle shape of the fibroblasts was observed with a Giemsa stain.

The statistical analysis was evaluated by nonparametric Mann–Whitney test ($p < 0.05$).

Subconfluent primary culture formation for the quantification of the percentage of fibroblasts expressing c-Fos and c-Myc

A total of 5×10^6 viable BM light-density mononuclear cells were placed in 25-cm^2 tissue culture flasks (Corning) that contained 10 ml of supplemented α-medium and 20% FBS. 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 until subconfluence (85–90%). From the initiation of the experiment until day 60, or until the cells reached subconfluence, the medium was changed every 7 days. When subconfluent, the fibroblasts were arrested for 72 h and then washed twice with PBS. They were isolated by a trypsin-EDTA treatment (0.05–0.02% in PBS). A total of 100,000 MSC were incubated in 500 μl of PBS with 10% FBS for 60 min at a 37°C temperature in a plastic chamber slide (8 wells/slide; Labtec de Nunc Co). Controls without stimulation were held. After this period of incubation, the wells were washed with PBS, dried at room temperature, and then stored at −20°C until used.

The quantification of the percentage of fibroblasts expressing both proteins was realized by the immunocytochemical technique (Histostain-Plus kit, alkaline phos-

phatase-conjugate/Fast Red chromogen; Zymed). Specific mAbs were anti-human c-Fos (dilution 1:10, IgG_1 isotype; Serotec) and anti-human c-Myc (dilution 1:50, IgG_1 isotype; Zymed). Negative controls were accomplished (mouse primary Ab isotype control, or without first Ab). The samples were thawed at room temperature and fixed with 100% methanol to increase the performance of Ab diffusion. Once dried, the samples were rehydrated in water for 5 min and then for another 10 min with TBS (5 mM Tris, 0.015 M NaCl, pH 7.6) at room temperature.

Levamisol (1 mM; Zymed) was added to the chromogen solution to inhibit the alkaline phosphatase activity in the fibroblast membrane. Hematoxylin (Zymed) was used as a contrast solution, and aqueous glycerol vinyl alcohol as mounting solution. The fibroblastic nature of the adherent cells that composed the cultures was demonstrated as described before. The statistical analysis was evaluated by a nonparametric Dunn's multiple comparison test ($p < 0.05$).

RESULTS

BM micrometastasis

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

CFU-F assay

As described in previous work, these patients continued to show a significant decrease in the CFU-F number, as shown in Fig. 1.

Percentage of subconfluent (85–90%) fibroblasts expressing receptors of PDGF, TGF-β, EGF, FGF, and IL-1 in BM primary cultures from LCP, BCP, and healthy patients

Regarding IL-1R-I, the results showed values in between the normal range in the percentage of subconfluent fibroblasts expressing this receptor in LCP and BCP (healthy patient = 100, LCP = 99.00 ± 0.25, and BCP = 98.6 ± 1.16). In contrast, the percentage of subconfluent fibroblasts expressing FGFR-I, EGFR, PDGFR-α, and TGF-βR-I-II-III showed diminished values in some cancer groups when compared to healthy patients. For each case, the results are shown in Figs. 2–7, respectively. Furthermore, a difference in the expression pattern of receptors per fibroblast was observed when comparing cancer patients and healthy patients. This pattern goes from maximum expression (++++) to minimum detectable expression (+), showing intensity of color of substratum per fibroblast. The cellular distribution pattern for each receptor is shown in Table 2.

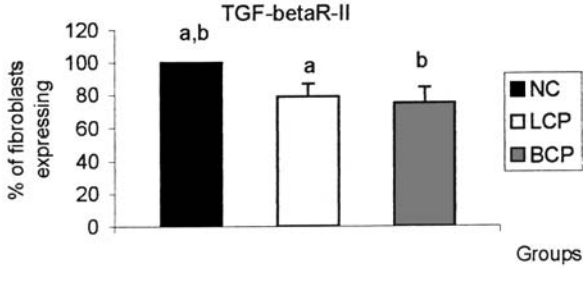

FIG. 6. Percentage of subconfluent fibroblasts expressing TGF-βR-II. Values are expressed as X ± SE. Statistical significance: a = $p < 0.006$, b = $p < 0.02$.

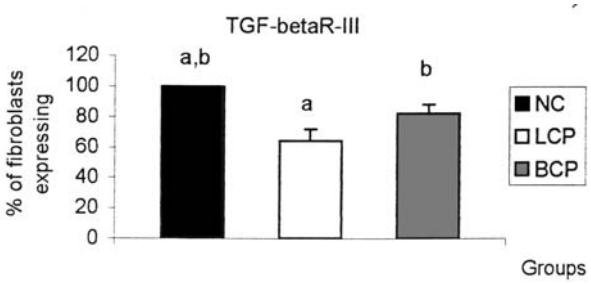

FIG. 7. Percentage of subconfluent fibroblasts expressing for TGF-βR-III. Values are expressed as X ± SE. Statistical significance: a = $p < 0.05$, b = $p < 0.02$.

Percentage of subconfluent fibroblasts expressing c-Fos and c-Myc in BM primary cultures from LCP, BCP, and healthy patients

Cultures with no FBS stimulation: A significant diminution of the percentage of fibroblasts expressing c-Fos in both cancer group's cultures studied was observed when compared to healthy patients. Nevertheless, when observing the percentage of fibroblasts expressing c-Myc, a diminution was also observed in both cancer groups, but the difference was only significant in the BCP group when compared to healthy patients.

Cultures with FBS stimulation: A significant diminution of the percentage of fibroblasts expressing c-Fos in both cancer group's cultures studied was observed when compared to healthy patients. When observing the percentage of fibroblasts expressing c-Myc, a diminution is also observed in both cancer groups but the difference is only significant in the LCP group when compared to healthy patients.

Nevertheless, if we analyze the percentage of increments regarding the basal level (without FBS stimulation) in c-Fos, the three groups reacted in the same way to serum stimulation. Regarding c-Myc, the percentage of increment after stimulation with FBS was greater in

Table 2. Expression Pattern of Receptors on Subconfluent Fibroblast Samples

	IL-1R type I	*FGF R type I*	*EGF R*	*PDGF R α-chain*	*TGF-β R*		
					Type I	*Type II*	*Type III*
NC	++++	+++	+++	++++	++++	++++	++++
LCP	++++/ ++(60%)[a]	+++/ +(50%)	++/ +(70%)	+++	+++/ ++(40%)	++	++
BCP	++++	++ +(60%)	++/	++++	++	+++/ ++(60%)	+++

[a]The parentheses must be interpreted in this way: for example, 60% of the samples assayed had ++ of expression pattern.

the BCP group than healthy patients, whereas the LCP had none (Table 3).

A minor level of expression of c-Fos and c-Myc/fibroblast was found in both groups of patients compared to healthy patients.

DISCUSSION

Experimental data indicated that stimulation of the initial proliferation of marrow stromal fibroblast progenitors or CFU-F is complex and requires participation of at least four growth factors: PDGF, bFGF, TGF-β, and EGF (7). Evidence from several model systems demonstrated the physiological importance of the coordination signal transduction to support cell proliferation, migration, and invasion in vivo. For example, the necessity of both EGF and PDGF to increase c-*fos* and c-*myc* expression on foreskin fibroblasts has been reported, supporting the idea that these two growth factors might act via a common intracellular pathway in the prereplicative phase of human fibroblasts (23). Moreover, the importance of bFGF (in the presence of serum) and EGF as potent mitogens for BM-CFU-F in a dose-dependent way have been described (7,24,25).

In the present study, we have reported a diminished percentage of subconfluent fibroblasts expressing PDGFR-α, FGFR-I, EGFR, and TGF-βR-I, II, and III in LCP and BCP compared to healthy patients. Furthermore, a diminished percentage of subconfluent fibroblasts expressing c-Fos and c-Myc was also described in these patients when compared to healthy patients. Simultaneously, a minor expression of these receptors per fibroblast and c-Fos and c-Myc per fibroblast was found in both groups of patients when compared to healthy patients.

In other words, the inefficient proliferative and confluence capacity of MSCs previously observed in CFU-F cultures from BM of untreated, advanced LCP and BCP might not only result from genetic alterations such as activation of oncogenes or inactivation of tumor suppressor genes, but also from altered production of, or responsiveness to, stimulatory or inhibitory growth and differentiation factors and their union to their specific receptors.

Table 3. Percentage of Subconfluent Fibroblasts Expressing c-Fos and c-Myc in BM Primary Cultures from LCP, BCP, and NC

	c-Fos		*c-Myc*	
	Without FBS	*With FBS*	*Without FBS*	*With FBS*
LCP	23.10 ± 9.06	39.44 ± 7.21 (16.3%)*	59.62 ± 10.56	54.33 ± 8.22 (0%)*
	a	c		e
BCP	20.93 ± 4.80	35.46 ± 3.29 (14.5%)*	46.48 ± 4.90	78.32 ± 8.52 (31.8%)*
	b	d	f	
NC	61.43 ± 2.56	72.83 ± 5.13 (11.4%)*	71.75 ± 3.27	90.61 ± 2.47 (18.4%)*
	a,b	c,d	f	e

Values are expressed as X ± SE.
*Percentage of increment.
Statistical significance: a,b,c,d,e,f = $p < 0.05$.

It is important to comment that even though the MSCs, the majority of fibroblasts from the patients we studied here were able to reach confluence, the time they needed was significantly longer than for the healthy patients (19). Our previous data support this fact; only 20% (6/30) of LPC-BM cultures and 11% (3/27) of BCP-BM cultures were able to reach confluence within the normal range (13–27 days), and only 60% of LCP and 78% of BCP were able to reach first confluence (19). The TGF-βR-III is known to be represented by CD105, which is well known as the endothelial antigen endoglobin and as SH2 antigen (26). The SH2 antigen is described to be a putative marker of mesenchymal stem cells and immature stroma progenitors. This fact is very relevant in the context of our results, because if these patients have less mesenchymal stem cells with $CD105^+$ they may not be able to go successfully through the proliferation and differentiation processes. This result might explain, in part, the decreased number of CFU-F found in BM cultures from untreated advanced LCP and BCP, because each CFU-F derives from a single mesenchymal stem cell (8).

Furthermore, the TGFβR-III is sought to act as a modulator of TGF-β interaction with the signal TGF-βR-I and II, thus affecting the ability of cells to respond to TGF-β isoforms. It is known that the loss of TGF-β responsiveness occurs in several types of cancer (27). This loss of responsiveness is typically the result of loss of TGF-βR expression. Inhibition of the cell cycle by TGF-β is thought to be mediated in part by the down-regulation of proliferative proteins, such as c-Myc and c-Fos (28).

All of these data support the idea that BM-MSC is regulated by and also regulates several levels of interrelations and forms a complex network. Thus, each one of the performers in this play is executing their important and necessary action for the success of the proliferation process, and so this proliferation process can not be taken as a series of partial events but as a whole. Further studies are necessary to evaluate the functional activity of all of these receptors that are under-expressed in these patient MSC and to know the mechanisms that could explain this deficiency.

Some authors have reported that a decrease of CFU-F number would imply less self-renewal capacity of mesenchymal stem cells in vivo and also a previous uncharacterized mechanism of bone diseases, such as osteoporosis or bone metastasis (29,30). These last two processes imply the necessary existence of an imbalance between bone formation, osteoclastogenesis, and bone resorption. Recent studies in our laboratory have shown a significant diminution of the osteogenic potential of MSCs regarding the possibility that these cells give rise to osteoblasts and osteocytes, as well as an inadequate mineralization matrix and calcium deposition in the osteogenic cultures in these advanced untreated BCP (31). These results are very important because many diseases that affect the BM have profound effects on bone formation, involving interactions between abnormal and normal marrow cells and those of bone (32).

Finally, although it seems clear that functional abnormalities in the BM-MSC (especially fibroblasts) exist in untreated advanced LCP and BCP, it is not known whether the level of such alterations of these MSC has a possible relationship with the future clinical evolution of these patients and if more modifications represent a worse prognosis.

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