Fibroblastic Colony-Forming Units and Levels of Tumor Necrosis Factor and Prostaglandin E2 in Bone Marrow Cultures from Patients with Advanced Lung Carcinoma

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Presented at the XVIII Latinoamerican Congress of Physiology Science, Montevideo, Uruguay, April 12–15, 1994; and at I Latinoamerican Congress of Cytokines, Santiago de Chile, Chile, November 14–17, 1994.

Financially supported by the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina.

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Received December 16, 1997; revision received May 21, 1997; accepted June 4, 1997.

BACKGROUND. Although alterations of the bone marrow (BM) fibroblast colony-forming cells are involved in the development of diverse hematologic disorders, these progenitors still have not been well characterized in patients with solid tumors.

METHODS. The incidence of fibroblast colony-forming units (CFU-F) was evaluated in the cultures of unseparated and fractionated light density BM mononuclear cells (MC) from 25 consecutive untreated lung carcinoma patients (LCP) and 16 normal controls (NC). Unseparated MC also were cultured in the presence of indomethacin (10^{-6} M). Finally, the authors evaluated the spontaneous production of prostaglandin E2 (PGE2) and tumor necrosis factor-α (TNF-α) in culture conditioned mediums of unseparated MC by radioimmunoassay and enzyme-linked immunoadsorbent assay methodology, respectively.

RESULTS. A decreased number of CFU-F was observed in unseparated and fractionated (adherent and nonadherent) light density MC cultures from LCP compared with NC. When unseparated MC of LCP were treated with indomethacin, a slightly increase in the number of CFU-F was found. Adherent MC (stromal cells) achieved confluence only in 44% of LCP primary cultures compared with 100% of NC. Overproduction of PGE2 and TNF-α was found in the conditioned mediums of LCP compared with the mean values obtained in NC ($P < 0.05$ and $P < 0.02$, respectively).

CONCLUSIONS. The lack of confluence and suppression of CFU-F in BM of LCP may be related to the increase production of PGE2 and TNF-α. Future investigation will allow the determination of how these modifications influence tumor cell growth and will prove if more alterations of the hematopoietic microenvironment imply a worse prognosis. ***Cancer*** **1997;80:1914–9.** © *1997 American Cancer Society.*

KEYWORDS: bone marrow fibroblast, prostaglandin E2, tumor necrosis factor-α, lung carcinoma.

Several reports have shown that most (80–90%) human bone marrow (BM)-adherent mononuclear cells (MC) are fibroblasts.[1,2] In 1974, Friedenstein et al.[3] introduced the concept of BM fibroblast colony formation. More recently, Castro Malaspina et al.[2] determined that such fibroblast colony-forming units (CFU-F) evolve from extremely adherent, nonphagocytic, nonproliferating, and relatively radioresistant progenitors. These stromal cells express fibronectin and Type I, III, and IV collagen and have low density and a wide distribution of sedimentation rates. Other authors[4,5] reported that CFU-F could be produced from the nonadherent populations of MC.

Conversely, CFU-F is the only clonal assay that is available for the stromal cell precursor, thus indicating that CFU-F reflect, at least in part, the proliferative capacity of stromal progenitors in human BM.[5] In addition, fibroblasts alone, and medium conditioned by them in a long term BM culture system, may be enough to support proliferation of committed progenitors of the myeloid and the erythroid series.[6,7]

Currently, in vitro studies have demonstrated functional abnormalities of the stromal cells in certain hemophaties such as congenital hypoplastic anemia, acute and chronic myelogenous leukemia, and myelodysplasia.[8–11] Although little is known regarding the effect of tumor growth, it was shown that chemotherapy and radiation can alter the functional integrity of the stroma.[12,13]

In preliminary studies,[14] we have observed that only a small number of CFU-F grew in BM collected from a high percentage of untreated patients with advanced lung and colorectal carcinoma. For this reason, the current study was especially undertaken to clarify the decrease in the cloning efficiency of fibroblast colony-forming cells. We evaluated the number of CFU-F obtained from unseparated and fractionated light density MC in untreated lung carcinoma patients (LCP). We also investigated the levels of prostaglandin E2 (PGE2) and tumor necrosis factor-α (TNF-α) present in the BM cultures. Both soluble factors studied have been shown to have an inhibitory effect on fibroblast growth.[15] Results suggest that the increase of PGE2 and TNF-α spontaneous release may play an important role in the regulation of the proliferative capacity of the fibroblastic progenitors.

MATERIALS AND METHODS

Patients

BM samples were obtained from 16 normal controls (NC) and 25 consecutive untreated patients with non-small cell advanced lung carcinoma of Stages IIIA, IIIB, and IV disease. The International Union Against Cancer TNM classification system was used. The NC and LCP were age-matched and gender-matched. Informed consent was obtained from all the individuals to participate in these studies. These studies were performed in accordance with the principles of the Declaration of Helsinki.

Collection and Preparation of BM Cells

BM samples were collected under local anesthesia from the posterior iliac crest into heparinized saline (25 units/mL) (Gibco, Grand Island, NY). First, we evaluated the composition of BM aspirate smears. Cells counts were performed after the smears were dried, fixed with 100% methanol, and stained with the Pappenheim technique. Afterward, aspirates were diluted 1:2 with phosphate-buffered saline (PBS) and were layered on Ficoll-Hypaque (density of 1.070 g/cm^3). After being centrifuged for 25 minutes at 1500 revolutions per minute (rpm), MC were harvested from the interface, washed twice in PBS and resuspended in an α medium (Gibco) containing 100 IU/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 25 μg/mL of amphotericin B (Gibco). The suspension was counted with 3% acetic acid solution and cell viability was determined by trypan blue dye exclusion.

The percentage of the fibroblastic nature of cells that comprised the unseparated MC ($\delta \leq 1070$ g/cm^3) was demonstrated by immunofluorescence method with a monoclonal antibody against the human β subunit of prolyl-4-hydroxylase (P4-H) (Dako Corporation, Carpinteria, CA). It is known that this active enzyme catalyzes the formation of 4-hydroxyproline in collagen. The antibody used reacts only with fibroblasts in normal and inflammatory tissue.[16,17] Cell smears were fixed with 50% methanol-50% acetone.

CFU-F Assay

CFU-F cells (fibroblasts precursors) were clonally assayed according to the method of Castro Malaspina et al.[2]; 5×10^6 viable unseparated MC, some of which were of a fibroblastic nature, were plated in 25-cm^2 tissue flasks (Corning Glass Works, Corning, NY) containing 10 mL of supplemented previously described α medium and 20% heat-inactivated fetal bovine serum (FBS) (cat. number 16000-044; Gibco). The cells were incubated at 37 °C and in a humidified atmosphere of 5% CO_2 in air for 7 days. After this period the nonadherent cells were removed and the medium was renewed. The cultures were returned to incubation for an additional 7 days. Finally, the medium was discharged and the adherent cells were washed twice with PBS, fixed with 100% methanol, and stained with Giemsa. Clones of >50 cells were scored as fibroblast colonies under a binocular dissecting microscope. In addition, adherent and nonadherent fractions of MC obtained after 30 minutes of incubation of 5×10^6 viable MC in T-25 culture flasks were cultured separately for CFU-F and the colonies were counted at Day 14. We chose 30-minute attachment in a plastic surface because other authors[2] proved that culture of adherent MC obtained after this time period showed a high number of CFU-F at Day 14 that remained stable even though the MC were adhered for longer time (60 and 90 minutes, respectively). Unseparated MC also were cultured in the presence of indomethacin (10^{-6} M; Sigma, St. Louis, MO). Moreover, in one sample of each subject, MC that remained attached after

60 minutes of incubation were trypsinized with a solution of trypsin-ethylenediamine tetraacetic acid (EDTA) (0.05–0.02%, respectively; Sigma) during 10 minutes at 37 °C and counted to evaluate the number of adherent MC detached after this treatment. It is well known that 80–90% of the normal adherent MC that have a fibroblastic nature are detached after this treatment.[2] This enzyme does not modify the viability of fibroblastic colony-forming cells.[2] This last experiment was performed after 60 minutes because adherence for this time period resulted in virtually complete separation of fibroblastic precursors.[2]

Simultaneously, some primary cultures (5×10^6 viable unseparated MC/25-cm^2 tissue culture flask) were allowed to grow until confluence to evaluate the possible relation between the number of CFU-F and the number of days that the adherent MC take to achieve confluence. The medium was changed every 7 days from the initiation of the experiment until Day 60 or until the cells reached confluence.

The fibroblastic nature of the adherent MC comprising the cultures was demonstrated by immunofluorescence staining with a monoclonal antibody against human fibronectin (gift from Dr. A. Korenbliht, Instituto de Ingeniería Genetica, Buenos Aires, Argentina) and cytochemical analyses with alkaline-phosphatase stain.

Preparation of Conditioned Medium

The unseparated MC (5×10^6) obtained from LCP or NC were plated in 25-cm^2 tissue culture flasks containing 10 mL of α medium, 12.5% FBS, 12.5% heat-inactivated horse serum (cat. number 16050-130; Gibco), and 1% antibiotic-antimycotic agent. The cells were incubated at 37 °C for 7 days. Conditioned mediums were obtained by centrifugation at 1000 rpm for 10 minutes and were frozen at −20 °C until use.

Determination of PGE2 and TNF-α

PGE2 was measured by radioimmunoassay methodology (RIA Kit, Dupont, Wilmington, DE) and TNF-α was quantified by immunoenzymatic methodology (enzyme-linked immunoadsorbent assay Kit, Dupont). Conditioned mediums were used pure and in 1:5 dilution for TNF-α and PGE2, respectively. The PGE2 assay detects levels between 2.50–250 pg/mL and TNF-α assay levels between 10–1000 pg/mL.

Statistics

All experiments were performed in duplicate. Statistical significance was evaluated by the Student's t test ($P < 0.05$). Values of the number of light density MC per 10 mL of BM aspirate were logarithmically transformed before comparison by one-way analysis of variance test due to the proportional relation between means and variances.

TABLE 1
Evaluation of the Cellular Composition of the BM from Untreated LCP and NC

	(No. of light density MC/10 mL BM aspirate) $\times 10^6$		
LCP	35.00	NC	30.46
	54.30		162.00
	37.80		277.00
	11.75		11.38
	37.50		23.75
	5.90		15.00
	9.30		20.00
	11.40		16.00
	11.56		15.00
	38.20		20.00
	60.90		16.00
	2.36		25.00
	5.35		10.00
	5.16		300.00
	4.10		40.00
	10.67		150.00
	11.30		
	11.60		
	22.28		
	30.00		
	5.00		
	27.00		
	20.00		
	10.00		
	21.73		
Mean ± SEM	20 ± 3[a]		70 ± 24[a]

BM: bone marrow; LCP: lung carcinoma patients; NC: normal controls; MC: mononuclear cells; SEM: standard error of the mean.
The values are expressed as individual values in both groups.
[a] $P < 0.05$.

RESULTS

Composition of BM Aspirate

The BM smears from LCP showed hypocellularity in all the hematopoietic and stromal progenitors, with the exception of erythroid series, which remained normal. Morphologic evidence of BM infiltration with neoplastic cells was not observed in LCP.

CFU-F Assay

Table 1 shows that LCP and NC had a significant difference in the number of light density MC/10 mL of BM aspirate. We also found similar values of the relative percentage of P4-H positive, light density MC (fibroblast colony-forming cells) between both groups. These last values were 10 ± 3% and 13 ± 6% for LCP and NC, respectively.

With regard to the cloning efficiency of CFU-F

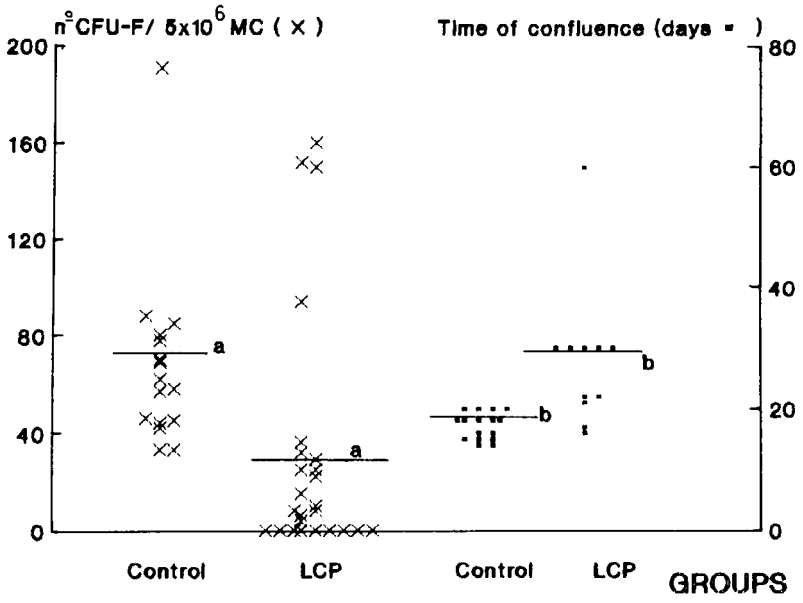

FIGURE 1. Evaluation of fibroblast colony formations and the ability of the stromal components to achieve confluence. The values are expressed as the mean of duplicate individual values in both groups, and the sample mean is indicated by a horizontal bar. Only 44% of lung carcinoma patients (LCP) had full confluent stromal layers until Day 60 of mononuclear cell culture whereas 100% of the normal controls had confluent adherent cell layers. a and b = $P < 0.01$. CFU-F: fibroblast colony-forming units.

(Fig. 1), we observed that BM of LCP reached lower values than that of NC ($P < 0.01$). Fig. 1 also shows that the BM adherent cells (majority fibroblastic progenitors) of only 44% of LCP could achieve confluence in primary cultures during the 60 days after the initiation of MC cultures. Furthermore, these patients presented with full confluent stromal layers after 28 ± 4 days as opposed to the NC, who took 17 ± 1 days ($P < 0.01$) (Fig. 1). In some of the samples of LCP that had a lack of confluence, colony incidence was very low or zero, and the remainder showed clusters as the only or predominant type of clonal growth (data not shown).

In addition, after 14 days, when the primary cultures treated with indomethacin were completed, the quantity of CFU-F in the 68% of LCP samples had increased compared with the quantity obtained in nontreated cultures (Table 2). Nevertheless, the mean value of the number of CFU-F in the LCP group continued to be lower than the NC value after indomethacin treatment (22 ± 3 and 72 ± 10 CFU-F/5 × 10^6 MC, respectively; $P < 0.001$). Moreover, Table 3 shows that CFU-F incidence in the cultures of adherent and nonadherent MC decreased in the BM of LCP compared with the values obtained in NC ($P < 0.03$ in the adherent fraction). However, after 60 minutes of 5 × 10^6 MC incubation, the cultures of LCP had a number of adherent MC/flask and a number of trypsin-EDTA sensitive, adherent MC within the NC range (Table 4).

Release of PGE2 and TNF-α from Unseparated MC

The levels of PGE2 and TNF-α released spontaneously in the conditioned mediums of LCP samples increased significantly compared with the mean values obtained in NC cultures ($P < 0.05$ and $P < 0.02$, respectively) (Table 5).

DISCUSSION

During the last 30 years, some studies, both in vivo and in vitro, have focused on the culture characteristics of BM hematopoietic progenitor cells from patients with solid tumors.[18,19] However, until now, we have not been able to find reports that study the structural and physiologic characterization of the hematopoietic microenvironment from untreated patients with solid tumors.

The results presented in this article show that BM from LCP had a low number of CFU-F compared with BM from NC. This defective proliferative potential of the fibroblastic progenitors might explain, in most of the cases, the lack or diminished ability of the adherent stromal components to achieve confluence.

As discussed earlier, stromal cell colonies could be produced from adherent or nonadherent populations of MC. In our case, both fractions of MC from LCP presented with a lower incidence of CFU-F compared with the values observed in NC. However, when the unseparated MC from LCP were incubated with indomethacin, the cultures showed a slight increase in the number of CFU-F compared with nontreated cultures. In both groups the cloning efficiency of CFU-F was very heterogeneous, regardless of the culture treatment and the type of MC (adherent or nonadherent) used.

Our study in LCP also shows that MC with a fibroblastic nature exhibit some of the properties of MC

TABLE 2
Evaluation of the Effect of Indomethacin Treatment on the Cloning Efficiency of Fibroblastic Progenitors

	LCP		NC	
	None	Indomethacin	None	Indomethacin
	0	10	70	69
	36	45	69	80
	15	1	33	53
	8	16	33	35
	0	20	57	60
	25	38	46	45
	94	43	43	50
	6	16	46	45
	152	50	55	68
	0	10	47	43
	8	16	76	78
	29	27	63	60
	22	30	81	85
	4	10	83	82
	32	20	191	199
	0	8	88	100
	0	5		
	10	10		
	0	8		
	0	15		
	160	40		
	0	15		
	150	42		
	0	15		
	25	35		
Mean ± SEM	31 ± 10[a]	22 ± 3[b]	68 ± 9[a]	72 ± 10[b]

LCP: lung carcinoma patients; NC: normal controls; SEM: standard error of the mean.
Fibroblast colonies are expressed as the number of fibroblastic colony-forming units/5×10^6 mononuclear cells.
The results of fibroblast colonies represent the mean of duplicate individual values in both groups.
[a] $P < 0.01$
[b] $P < 0.001$.

TABLE 3
CFU-F Incidence in the Adherent and Nonadherent MC Fractions of BM from LCP and NC

Group	No. of CFU/ 5×10^6 MC[a]	No. of CFU-F of adherent MC	No. of CFU-F of nonadherent MC
LCP	26 ± 13[b]	13 ± 9[c]	14 ± 6
NC	108 ± 29[b]	69 ± 19[c]	39 ± 12

CFU-F: fibroblastic colony-forming units; MC: mononuclear cells; BM: bone marrow; LCP: lung carcinoma patients; NC: normal controls.
Values are expressed as sample mean ± standard error of the mean.
[a] Addition of the number of fibroblastic colony-forming units (CFU-F) for the adherent and nonadherent mononuclear cell (MC) cultures. Both fractions of MC were obtained after 30 minutes of incubation of 5×10^6 MC in T-25 flasks, and cultured separately for CFU-F in 37 °C, 5% CO_2 humidified environment over 14 days. The number of adherent MC after 30 minutes of incubation in plastic was similar in both groups (data not shown).
[b] $P < 0.05$
[c] $P < 0.03$.

TABLE 4
Number of Adherent MC after 60 Minutes of Incubation in Plastic Surface and Evaluation of Trypsin-EDTA Sensitive AMC

Group	No. of AMC $\times 10^6$ Time of adherence (60 minutes)	No. of trypsin-EDTA sensitive AMC $\times 10^{6a}$
LCP	2.75 ± 0.88	1.51 ± 0.64
NC	1.63 ± 0.13	1.50 ± 0.22

MC: mononuclear cells; EDTA: ethylenediamine tetraacetic acid; AMC: adherent mononuclear cells; LCP: lung carcinoma patients; NC: normal controls.
The values are expressed as sample mean ± standard error of the mean.
[a] The mononuclear cells (5×10^6) that remained attached after 60 minutes of incubation of T-25 flasks were trypsinized with a solution of trypsin ethylenediamine tetraacetic acid (0.05–0.02%, respectively) and counted to evaluate the number of fibroblastic precursors detached after this treatment.

TABLE 5
PGE2 and TNF-α Spontaneous Release in Conditioned Medium of MC Cultures from LCP and NC

Group	PGE2 (pg/mL)	TNF-α
LCP	108 ± 28[a]	294 ± 10[b]
NC	32 ± 15[a]	260 ± 6[b]

PGE2: prostaglandin E2; TNF-α: tumor necrosis factor-α; MC: mononuclear cells: LCP: lung carcinoma patients; NC: normal controls.
Values are expressed as sample mean ± standard error of the mean.
Prostaglandin E2 and tumor necrosis factor-α were measured by radioimmunoassay and enzyme-linked immunoadsorbent assay, respectively.
[a] $P < 0.05$
[b] $P < 0.02$.

derived from NC such as adherence, sensitivity to trypsinization, and the presence of β subunit of P4-H antigen.

Conversely, to clarify some mechanism of the decrease of fibroblast proliferative potential, we have evaluated some of the soluble factors that regulate CFU-F. Stromal MC secrete a variety of substances capable of regulating several fibroblast functions such as proliferation and collagen synthesis.[20] The stimulatory or inhibitory effect of these mediators may vary depending on the quantity of the secreted factor, the type of fibroblastic progenitor cultured, the area from which the sample was aspirated, and the fibroblast passage number.[20]

Our results show high levels of PGE2 and TNF-α in untreated BM culture conditioned mediums of LCP compared with NC values. PGE2 has an inhibitory effect on fibroblast proliferation in serum supplemented culture, and this inhibition is correlated with an in-

crease in intracellular cyclic adenosine monophosphate levels that are reversible with inhibitors of cyclooxygenase-like indomethacin.[20,21] Our results with CFU-F obtained in some patients using unseparated MC cultures treated with indomethacin are in accordance with this observation. However, some patients had low levels of PGE2 and had a significant decrease of fibroblast proliferation and a lack of confluence capacity. This last observation could be due to the inhibitor effect of TNF-α or another suppressing factor. Kovacs [15] reported that TNF-α acts as both a growth inducer and inhibitor. At low concentrations it stimulates fibroblast proliferation and at higher levels it blocks growth triggered by FBS or other cytokines such interleukin-1 and platelet-derived growth factor. It also is feasible that elevated levels of TNF-α released by stromal macrophages could increase the PGE2 production by fibroblasts or macrophages.[22,23] Therefore, the previous results support the theory that spontaneous overproduction of TNF-α and PGE2 produced by stromal cells can decrease the growth of fibroblast colonies in the majority of LCP. Further studies are required to clarify the complex process involved in fibroblast proliferation inhibition or decrease.

Finally, although it appears clear that functional abnormalities in the fibroblast progenitors exist in LCP, it is not known to what extent such modifications contribute to the development and/or progression of this tumor.

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