PERCUTANEOUS AUTOLOGOUS BONE-MARROW GRAFTING FOR NONUNIONS

INFLUENCE OF THE NUMBER AND CONCENTRATION OF PROGENITOR CELLS

BY PH. HERNIGOU, MD, A. POIGNARD, MD, F. BEAUJEAN, MD, AND H. ROUARD, MD

Investigation performed at the Service de Chirurgie Orthopédique, Hôpital Henri Mondor, Creteil, France

Background: Bone marrow aspirated from the iliac crest contains progenitor cells that can be used to obtain bone-healing of nonunions. However, there is little available information regarding the number and concentration of these cells that are necessary to obtain bone repair. The purpose of this study was to evaluate the number and concentration of progenitor cells that were transplanted for the treatment of nonunion, the callus volume obtained after the transplantation, and the clinical healing rate.

Methods: Marrow was aspirated from both anterior iliac crests, concentrated on a cell separator, and then injected into sixty noninfected atrophic nonunions of the tibia. Each nonunion received a relatively constant volume of 20 cm^3 of concentrated bone marrow. The number of progenitor cells that was transplanted was estimated by counting the fibroblast colony-forming units. The volume of mineralized bone formation was determined by comparing preoperative computerized tomography scans with scans performed four months following the injection.

Results: The aspirates contained an average (and standard deviation) of 612 ± 134 progenitors/cm^3 (range, 12 to 1224 progenitors/cm^3) before concentration and an average of 2579 ± 1121 progenitors/cm^3 (range, 60 to 6120 progenitors/cm^3) after concentration. An average total of 51 × 10^3 fibroblast colony-forming units was injected into each nonunion. Bone union was obtained in fifty-three patients, and the bone marrow that had been injected into the nonunions of those patients contained >1500 progenitors/cm^3 and an average total of 54,962 ± 17,431 progenitors. The concentration (634 ± 187 progenitors/cm^3) and the total number (19,324 ± 6843) of progenitors injected into the nonunion sites of the seven patients in whom bone union was not obtained were both significantly lower (p = 0.001 and p < 0.01, respectively) than those in the patients who obtained bone union. The volume of the mineralized callus measured at four months on the computerized tomography scans of the patients who had union ranged from 0.8 to 5.3 cm^3 (mean, 3.1 cm^3). There was a positive correlation between the volume of mineralized callus at four months and the number (p = 0.04) and concentration (p = 0.01) of fibroblast colony-forming units in the graft. There was a negative correlation between the time needed to obtain union and the concentration of fibroblast colony-forming units in the graft (p = 0.04).

Conclusions: Percutaneous autologous bone-marrow grafting is an effective and safe method for the treatment of an atrophic tibial diaphyseal nonunion. However, its efficacy appears to be related to the number of progenitors in the graft, and the number of progenitors available in bone marrow aspirated from the iliac crest appears to be less than optimal in the absence of concentration.

Level of Evidence: Therapeutic Level III. See Instructions to Authors for a complete description of levels of evidence.

The osteogenic capacity of bone marrow was first demonstrated in rabbits as early as 1869 by Goujon[1]. Since the 1960s, some authors[2-4] have shown that osteogenic stem cells in bone marrow are responsible for the biological efficacy of cancellous bone. This capacity has already been exploited, by several investigators[5,6], to reinforce the osteogenic properties of bone allograft by mixing the graft with bone marrow removed during surgery. In animal experiments, Connolly et al.[7] demonstrated a positive correlation between bone-marrow osteogenic capacity and cell concentration, and nonunions have been treated successfully clinically with autologous bone-marrow grafting alone[8,9]. However, the authors of

the clinical studies did not report the number of connective-tissue progenitor cells that were transplanted, and we are not aware of any study indicating the number of progenitor cells required to obtain bone-healing in the treatment of non-unions in humans. Furthermore, only limited clinical experience with the use of intraoperative centrifugation of marrow for bone-grafting has been reported.

The purpose of the present study was to evaluate the number and concentration of progenitor cells that were transplanted for the treatment of fracture nonunions, the callus volume after transplantation of the concentrated bone marrow, and the rate of clinical union.

Materials and Methods

Operative Technique

Marrow Aspiration

Marrow was aspirated from the two anterior iliac crests with the patient under general anesthesia. After deep insertion of a beveled needle (6 to 8 cm in length and 1.5 mm in internal diameter) into spongy bone, the marrow was aspirated into a 10-mL plastic syringe. At a given depth, the needle was turned 45° to reorient the bevel during successive aspirations, so that the largest possible space was aspirated. After one full turn, the needle was moved 1 cm toward the surface through the same insertion site, and aspirations were again performed, with the needle always turned 45° after each aspiration. The marrow was aspirated in small fractions (4 mL) to reduce the degree of dilution by peripheral blood[10]. Three, four, or five perforations were made, through the same skin opening, into the iliac crest, with the perforations spaced approximately 2 cm from each other to avoid dilution by aspiration in the previous hole. All aspirates were pooled in plastic bags containing an anticoagulant solution (citric acid, sodium citrate, and dextrose). Pooled aspirates were then filtered to separate cellular aggregates and fat (Hemoset NSR LP; B-Braun, Bethlehem, Pennsylvania).

Concentration

Concentrated buffy coat was obtained after a five-minute centrifugation at 1200 g on a cell separator (Cobe 2991; Gambro BCT, Lakewood, Colorado). This centrifugation forces the polynuclear cell layer, which is heavier because of the volume of its nuclei, to the periphery, where it can be collected and separated from the remainder. The lighter layer with anuclear red cells is found in the center and is also removed. The buffy coat contains progenitor cells but also other mononuclear cells, and some of these other cells may be a source of angiogenic or osteogenic cytokines[11] with a clinical effect. This centrifugation method reduces a 300-mL bone-marrow aspirate to a concentrated buffy coat of about 50 mL, which is poured into a syringe for injection.

Intraosseous Reinjection

A trocar identical to that used to aspirate the marrow was placed both in the nonunion gap and around the bone ends. The tip of the trocar was positioned with use of an image inten-

sifier (Fig. 1). The fibrous tissue of the nonunion site was not removed or disturbed. The marrow was injected slowly at a rate of about 20 mL/min. After injection, the trocar, with the stylet in place, was gradually withdrawn with small oscillating motions (backward and forward) to fill in the path of the trocar.

Patient Demographics

Sixty patients with an established nonunion of the tibial shaft were treated with this technique at the same center between 1990 and 2000. Thirty-eight patients were male. Twenty-eight patients had comorbidities: fifteen had a history of tobacco use, eight had a history of alcohol abuse, three had diabetes, and two had used a pharmaceutical agent affecting bone marrow physiology. Seventeen fractures were in the proximal part of the tibia, twelve were in the distal part of the tibia, and thirty-one were in the midpart of the tibial shaft. The patients ranged in age from eighteen to seventy-eight years, with a mean age of forty years. There were twelve isolated, closed, low-energy fractures of the tibial shaft, which had been treated nonoperatively with a plaster-of-Paris cast. There were forty-eight open fractures, which had been treated with external fixation (a monolateral frame was used for four cases and a biplanar frame, for forty-four). The majority (fifty-seven) of the patients underwent definitive fracture fixation with external fixation or a cast immediately (within twenty-four hours) after the injury. Three patients underwent changes in the external fixation during the first week. All patients with an open

Fig. 1

The trocar is positioned in the nonunion under fluoroscopy.

fracture received routine soft-tissue management, including débridement, irrigation, and definitive wound closure, immediately or within three days. According to the Gustilo-Anderson classification of open injuries[12], thirty-six fractures were type I, eight were type II, two were type IIIA, one was type IIIB, and one was type IIIC. The type-IIIB open fracture required flap coverage, and the type-IIIC open fracture required a vascular repair. Of the forty-eight open fractures, seven (five type I, one type IIIA, and one type IIIC) had already been treated, in another center, with an autologous cancellous bone graft between the fourth and seventh month after the injury. The graft was harvested with an open procedure from the anterior iliac crest in five patients and from the posterior iliac crest in two at an average of four months (range, three to six months) before the aspiration of the bone marrow graft; the aspiration was done at a minimum of 2 cm from the area of the previous graft harvest.

The definition of nonunion was a failure of the fracture to heal in six months in a patient in whom progressive repair had not been observed radiographically between the third and sixth month after the fracture. All nonunions were considered atrophic because they showed very little callus formation. The time between the fracture and the bone marrow injection ranged from six to twelve months (average, eight months and three weeks).

At the time of the bone marrow grafting, the nonunions were considered to be not infected according to preoperative assessment for the presence of systemic infectious parameters. The absence of infection was confirmed postoperatively by the results of culture of aspirate obtained from the nonunion site just before the bone marrow injection.

The displacement of the bone fragments at the time of the bone marrow grafting was measured, on anteroposterior and lateral radiographs, as a percentage of the width of the bone at the level of the fracture. Fragment displacement ranged from 0% to 20%, with an average of 6%. The maximum gap between the fragments was always <5 mm.

Management of the Nonunions Before and After Bone Marrow Grafting

Anteroposterior and lateral radiographs and a computerized tomography scan were made preoperatively. Postoperatively, radiographs were made at three and four weeks to assess the appearance of the callus and then every month thereafter, until bone healing occurred, to monitor the progression of the callus.

The volume of mineralized callus was calculated from measurements made on the computerized tomography scans. The area of callus resulting from the injection was considered to be the sector of new bone formation between the time of the preoperative computerized tomography and the time of the computerized tomography performed four months postoperatively. The protocol for the computerized tomography scanning consisted of 3-mm-thick sections over a length of 6 cm at the level of the nonunion (3 cm proximal and 3 cm distal). The level of each cut was controlled visually to be certain that the measurements on the preoperative and follow-up

scans were made at the same level. The images were analyzed with use of a set of custom algorithms to determine pixel intensity and the extent of mineralized bone surface at the fracture site. Because unmineralized tissue cannot be evaluated accurately with computerized tomography, it was not analyzed in this study. Although the computerized tomography scan is two-dimensional, it provides information from a three-dimensional slab. For each slice, the volume of callus was calculated by multiplying the area of callus by the slice thickness. The total volume of callus was the sum of the individual volumes of each slice. Computerized tomography was not used to determine union or as a guide to ascertain when to allow full weight-bearing.

The only therapeutic intervention performed in the present study was percutaneous injection of bone marrow. The same external fixation (for the open fractures) or plaster-cast immobilization (for the closed fractures) was used after the bone marrow grafting. All of the patients were treated with a standard protocol during the first month following the injection. As they had atrophic nonunion and mobility at the fracture site, weight-bearing was not allowed during the first month following the injection to avoid mechanical disruption of the tissue-regeneration and bone-healing processes. After one month, if (and only if) callus was observed on radiographs, partial weight-bearing was allowed with the plaster cast or external fixation in place. There was a one-month transition period between the beginning of partial weight-bearing and that of full weight-bearing. At the end of that month, if the patient had no pain and there was cortical bridging or disappearance of the fracture lines on at least three of the four cortices viewed on the anteroposterior and lateral radiographs, the plaster cast or the external fixation was removed.

The treatment was considered to be a success when there was definite radiographic evidence of fracture union and fulfillment of the clinical criteria of healing within six months after the autologous bone-marrow grafting. The clinical criteria of healing included full weight-bearing and no tenderness at the fracture site on palpation. When a patient did not have bone-healing six months after the bone marrow grafting, a secondary intervention to promote fracture union was proposed to him or her and the treatment was considered a failure. Each patient was followed for at least three years after the bone marrow grafting.

Bone Marrow Analysis

To measure the number of connective-tissue progenitor cells that were transplanted, we used the fibroblast colony-forming unit (CFU-F) as an indicator of stromal cell activity. The fibroblast is not an osteogenic cell but, according to the theory of pluripotential cell lines, osteocytes develop from colony-forming-unit progenitor cells in the marrow[11,13-15]. There seems little doubt that these colonies are clonal (i.e., originate from a single cell), and in this paper the terms "stem cell," "connective-tissue progenitor cell," "progenitor," and "CFU-F" will be considered synonymous. The aggregate of the marrow was cultured in vitro before and after concentration in order to de-

termine how much the concentration process altered the number of stem cells in the sample. The number of nucleated cells was counted with use of a standard Malassez hemocytometer (Polylabo, Strasbourg, France). Cells were washed once and resuspended in Hanks balanced salt solution without Ca++ or Mg++. Buffy coats were collected after centrifugation of the aspirates at 1200 g for ten minutes.

For the fibroblast colony-forming units (CFU-F), quadruplicate aliquots of 2×10^6 cells were inoculated in 25-mL^2 tissue-culture flasks containing 10 mL of culture medium supplemented with 20% fetal calf serum, 1% L-glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL). The culture flasks were placed in a humidified incubator with 5% CO_2 and maintained at 37°C. The growth medium was completely renewed every three to four days, and the cultures were evaluated on the tenth day. Fibroblast colonies were Giemsa-stained and were counted under an inverted microscope at 25× magnification. An aggregate of cells containing more than fifty fibroblasts was scored as a colony. Results were expressed as the mean number of fibroblast colony-forming units per 10^6 bone marrow cells. The fibroblastic nature of the colonies was demonstrated by immunofluorescence staining with antibodies against fibronectin and type-I and III collagen.

Statistical Methods

Data are reported as the mean and standard deviation, and the significance level was set at a probability value of <0.05. The outcome variables were the success of the treatment, the volume of the mineralized callus at four months, and the time needed to obtain union after the bone marrow grafting. The therapeutic factors that could influence the outcome variables were the total number and the concentration of fibroblast colony-forming units injected at the nonunion site. The patient and fracture variables included age, sex, associated comorbidities, fracture displacement, and type of open injury according to the classification of Gustilo and Anderson. A multivariate analysis was conducted to evaluate the relationship between the outcome and the set of variables. Correlations between the outcome variables and the cell factors were determined with use of the Spearman correlation test. The nonparametric Mann-Whitney U test was used to identify the significance of the differences between groups. The chi-square test was used to identify trends within groups with categorical variables.

Results

None of the patients had complications during anesthesia; in particular, no patient had a decrease in oxygen saturation or a change in pulse or blood pressure during the procedure. A compartment syndrome did not develop in any patient after injection of the bone marrow. There were no infections, hematomas, or chronic pain at the site of the bone marrow injection.

Patient and Bone Marrow Variables (Table I)

An average of 306 ± 24 mL of marrow was aspirated from the two iliac crests of each patient. The number of nucleated cells obtained from the individual patients ranged from 1 to 24 million/mL, with a mean of 18 ± 7 million/mL. The mean number of fibroblast colony-forming units per one million nucleated cells obtained from the individual patients ranged from 7 to 51, with a mean of 33 ± 8. The number of nucleated cells was found to decrease significantly with age (Spearman test, p = 0.03), but, with the numbers available, no significant difference between men and women was found (p = 0.26).

TABLE I Patient Demographics

Characteristic	No.	Bone Marrow Variables*		Progenitors in the Graft*	
		Nucleated cells (million/mL)	Prevalence of CFU-F (colonies/ million nucleated cells)	No. × 10³	Concentration (no./cm³)
All patients	60	18 ± 7	33 ± 8	51 ± 38	2579 ± 1121
Male	38	19 ± 8	35 ± 7	53 ± 41	2728 ± 1243
Female	22	16 ± 6	30 ± 10	48 ± 37	2321 ± 1024
Comorbidities	28	16 ± 10	29 ± 12	45 ± 43	2217 ± 985
No comorbidities	32	20 ± 6	37 ± 6	57 ± 32	2896 ± 1192
Closed fractures	12	17 ± 11	29 ± 9	48 ± 22	2140 ± 341
Open fractures					
I	36	18 ± 6	30 ± 8	51 ± 27	2331 ± 1204
II	8	20 ± 7	47 ± 5	52 ± 19	3927 ± 500
III	4	19 ± 12	46 ± 7	53 ± 20	3438 ± 300
Age					
>40 yr	30	15 ± 6	32 ± 7	49 ± 33	2407 ± 972
<40 yr	30	21 ± 9	34 ± 9	52 ± 22	2752 ± 1425

*The values are given as the mean and standard deviation.

There was no significant change in the prevalence of progenitor cells with increasing age (p = 0.12) and, when men and women were analyzed separately, there was no significant change with age in men (p = 0.28); however, the prevalence of progenitor cells was observed to decrease significantly with increasing age in women (p = 0.04).

An average of 1 CFU-F/30 × 10³ bone marrow nucleated cells was obtained in the samples incubated in vitro. The bone marrow obtained by aspiration from the iliac crests contained an average of 612 ± 134 progenitors/cm³ (range, 12 to 1224 progenitors/cm³). After concentration, the bone marrow contained an average of 2579 ± 1121 progenitors/cm³ (range, 60 to 6120 progenitors/cm³). A mean of 20 cm³ (range, 17 to 22 cm³) of bone marrow graft was injected into each nonunion site. The average total number of fibroblast colony-forming units injected into each nonunion site (i.e., the product of the nucleated cells and the prevalence of progenitors in the bone marrow graft obtained after concentration) was 51 × 10³ (range, 1200 to 122 × 10³).

Analysis of the total population demonstrated that age had no significant effect on the total number of progenitor cells received by each patient (p = 0.08). Also, when men and women were analyzed separately, age was found to have no significant effect on the number of cells received by the men; however, increasing age was found to be associated with a significant decrease in the total number of progenitors received by the women (p = 0.04). With the number of patients available, the comorbidities of smoking, alcohol abuse, diabetes, and use of pharmaceutical agents were not associated with significant changes in the population of cells that were harvested.

Outcomes of Management of the Nonunions

Nonunion outcome variables were defined as the success of the treatment; the healing time; the volume of callus; and the change in displacement, shortening, or angulation during bone-healing.

Bone union was obtained in fifty-three of the sixty patients, with the callus typically appearing on radiographs between the third week and the second month after the injection. Radiographic evidence of fracture union (Fig. 2) was observed at an average of twelve weeks (range, four to sixteen weeks). The volume of the mineralized callus measured at four months on the computerized tomography scans of these fifty-three patients ranged from 0.8 to 5.3 cm³, with a mean value of 3.1 cm³. During healing after the bone marrow grafting, shortening ranged from 0 to 25 mm, with an average of 5.4 mm. Fifty nonunions healed with <15 mm of shortening, and three healed with >15 mm of shortening. During healing, forty-nine patients did not have an increase of 3° in angulation in the frontal plane, and fifty patients did not have an increase of 3° in the sagittal plane. Four patients had an increase in angulation of between 3° and 7° in the frontal plane, and three patients had an increase in angulation of between 3° and 8° in the sagittal plane. Forty-six nonunions healed with no more displacement in any plane, and seven had an increase in displacement (ranging from 5% to 10%) during healing.

Of the sixty patients, seven did not have union, with the volume of mineralized callus in those patients measuring <0.5 cm³ on the computerized tomography scan. Three of the seven patients had an increase in angulation of >10° and an increase in displacement of >20%. These seven patients required additional surgery to achieve healing. Intramedullary nailing was performed in three; open bone-grafting, in three; and fibular osteotomy, in one.

Statistical Analysis

Success of treatment: Of the variables that were explored with multivariate analysis, the number of transplanted cells was deemed to be the most relevant to the outcome (Figs. 3 and 4). As the volume of the graft was relatively constant (average, 20 cm³; range, 17 to 22 cm³), the concentration of transplanted cells also appeared to be relevant to the outcome. The bone marrow grafts used for the fifty-three patients who subsequently had bone union contained a mean of 2835 ± 1160 progenitors/cm³ and a mean of 54,962 ± 17,431 progenitors in total, and all of the grafts in these patients contained >1500 progenitors/cm³. The grafts used in the seven patients in whom the treatment failed contained a significantly lower concentration (mean, 634 ± 187 progenitors/cm³, p = 0.001) and total number (mean, 19,324 ± 6843, p < 0.01) of progeni-
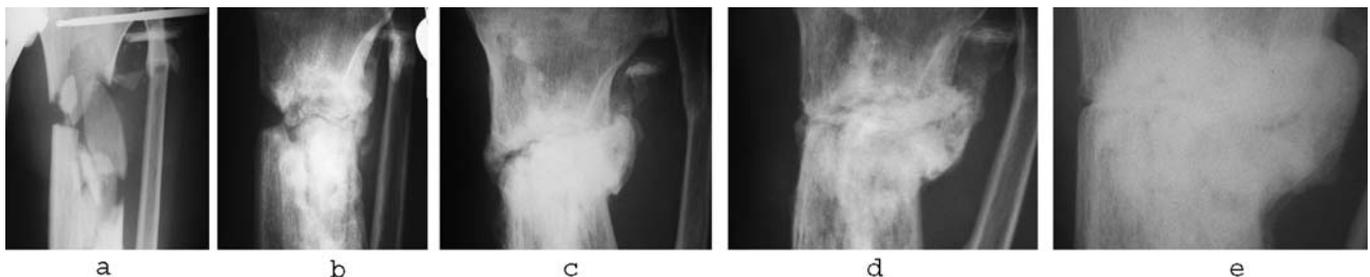

Fig. 2

Anteroposterior radiographs of a twenty-five-year-old patient who had sustained a type-I open fracture. The radiographs were made at the time of fracture (a); at the time of nonunion, before injection of autologous bone marrow (b); at one month after bone marrow injection, at which time the patient was allowed to begin partial weight-bearing (c); at two months after bone marrow injection (d); and at three months after bone marrow injection, at which time the external fixation was removed (e).
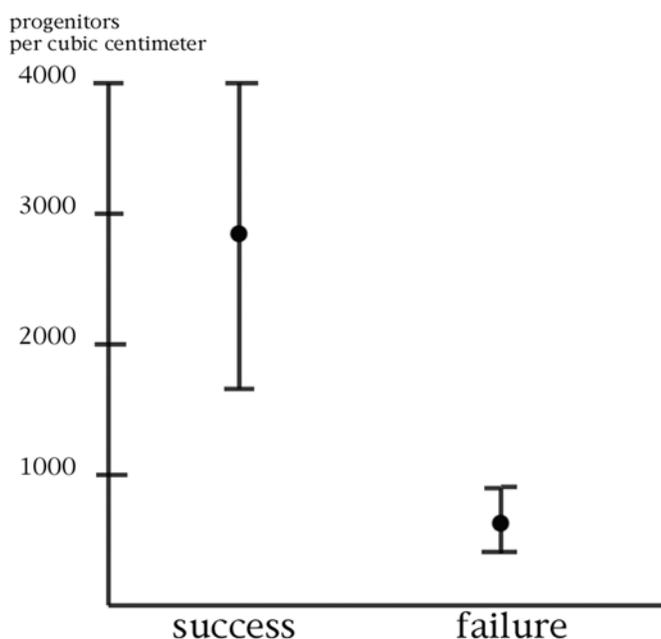
Fig. 3

Success and failure of treatment as a function of the concentration of
progenitor cells per cubic centimeter in the graft.

tor cells compared with the patients in whom the treatment
was successful. All seven patients with failure of union had
been treated with a bone marrow graft that contained <1000
progenitors/cm³ and <30,000 progenitors in total. With the
numbers available, the age, sex, and comorbidities of the pa-
tients did not significantly affect the success of the treatment
(p > 0.05).

Healing time: There was a negative correlation between
the time needed to obtain union and the concentration of fi-
broblast colony-forming units in the graft (Rs [Spearman cor-
relation test] = –0.2, p = 0.04). Fracture type also had a
significant relationship with the time to union (p = 0.03), with
type-II and type-III open fractures taking longer to heal (aver-
age, fourteen weeks) than closed fractures and type-I open
fractures (average, eight weeks). In addition, the location of
the fracture had a relationship with the time until healing (p =
0.03), with the distal fractures taking longer to heal (average,
thirteen weeks) than the proximal fractures (average, nine
weeks). Finally, there was a significant relationship between
comorbidities and the time to union (p = 0.04): the twenty-
eight patients with one or more comorbidities had a longer
time until healing (average, fourteen weeks) than the other
patients (average, 10.5 weeks).

Volume of callus: There was a positive correlation be-
tween the volume of mineralized callus at four months and
the number and concentration of fibroblast colony-forming
units in the graft (correlation coefficient, 0.3 and 0.6; p =
0.04 and 0.01, respectively). With the numbers available, age,
sex, comorbidities, and fracture type had no significant in-
fluence on the volume of the callus (p = 0.61, 0.24, 0.34, and
0.28, respectively).

Discussion

During the past two decades, numerous techniques have
been developed to treat fracture nonunions, ranging from
invasive interventions (including internal fixation with the use
of bone graft or bone graft substitutes) to noninvasive proce-
dures (ultrasound and pulsed electromagnetic fields). The per-
cutaneous technique of autologous bone-marrow grafting that
we used is a minimally invasive alternative.

Our study showed that percutaneous autologous bone-
marrow grafting is a safe treatment for uninfected atrophic
nonunions of the tibial diaphysis, as we encountered no local
or systemic complications. One theoretical criticism of this
technique is that there is a risk of fat embolism during the in-
jection of the bone marrow into the nonunion site. However,
in our study, the bone marrow aspirates were filtered to sepa-
rate the marrow and fat, and none of the patients had compli-
cations during anesthesia.

Fifty-three of the sixty nonunions healed, which con-
firms the effectiveness of this technique for the treatment of
atrophic nonunions[8,9,16,17]. Historically, resection of the fibrous
tissue at the nonunion site combined with mechanical stabili-
zation has been described as being essential for the treatment
of an atrophic nonunion[18]. In this series, the trocar was not
used to remove the intervening callus or fibrous tissue. The fi-
brous tissue interposed between the bone ends ossified after
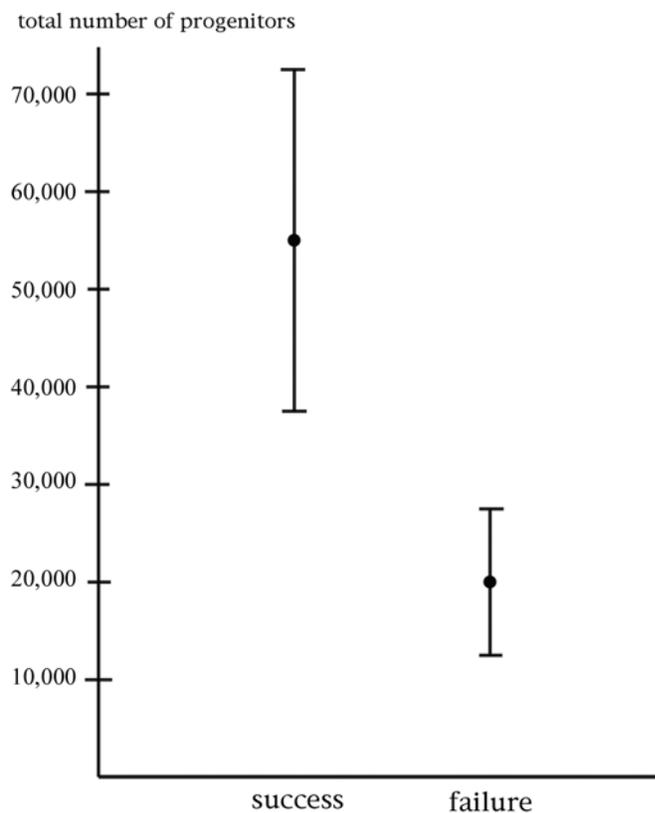

Fig. 4

Success and failure of treatment as a function of the total number of
progenitor cells injected at the nonunion site.

the injection of the bone marrow. It is difficult to explain the exact mechanism that allows the transformation of fibrous tissue into callus. Bone marrow was injected both in the nonunion gap and around the bones. It is not possible to know whether the injected marrow was able to convert the fibrous tissue into bone or if the interposed tissue was transformed into bone only after the bridging callus (obtained from the graft around the bone) stopped micromotion at the nonunion site and allowed union of the gap.

Like all techniques, this new option of bioactive cell stimulation has its limitations, one of which is that it has not been evaluated in the presence of internal fixation (plates or intramedullary nails). One potential weakness of the present study is the absence of a cohort with a placebo treatment such as injection of saline solution. Also, the cell counts were determined retrospectively; thus, we cannot determine if the technique should have been used as the sole treatment method. Percutaneous injection of bone marrow cannot be used when there is pre-existing angular deformity or shortening, both of which require direct access to the nonunion site. As the volume of callus obtained with this technique is limited, the fracture fragment gap size and displacement should be limited as well.

Another important finding of this study is the relationship between the volume of the callus and the number of progenitors in the graft. The addition of bone-marrow-derived cells has been shown to enhance bone-healing in animals. There are limited data on the number of progenitors that are resident in bone marrow grafts in humans[19]. The variability in the osteogenic potential from patient to patient is a limitation of the technique, and little is known about the extent to which these cells are susceptible to activation for bone repair after they are implanted. Because we initially had no data on the number of cells necessary to obtain bone-healing, the volume of the aspirate and volume of the transplanted graft were similar for all of the patients. In this series, the number of progenitors was determined retrospectively, and there was variation among the patients.

Differences among connective-tissue progenitors harvested from various individuals are beginning to be understood. These differences depend on many variables, such as age, gender, and local and systemic disease[10,19-22], and the variability in the osteogenic potential from patient to patient represents a limitation of the technique. One of the challenges in the operating room for the surgeon using this technique could be the evaluation of the number of cells obtained by aspiration. Bone marrow cellularity declines with age, and there is also a decrease in the prevalence of connective-tissue progenitors with increasing age[23,24], even if this was not evident in our small series of patients. However, as can be observed by examining the data in our Table I and the information in other reports[21], age and gender account for only a fraction of the variation; thus, connective-tissue progenitors can be obtained by bone marrow aspiration from patients of all ages. It may be useful for surgeons to know the cellularity of the bone marrow when operating on older patients. The number of progenitors can be determined only with a culture, but the quantity of medullary nuclear cells can be evaluated in the operating room (if neces-

sary) by the equation presented in the Appendix. Since the total number of progenitors represents the product of the nucleated cells and the prevalence of progenitors in the aspirate, a decline in the number of nucleated cells can be corrected by an increase in the volume of aspiration. However, a larger volume of aspiration decreases the concentration of progenitor cells because of dilution with peripheral blood.

Still another important observation in this study was the influence that the concentration of bone marrow by centrifugation had on the results. The seven patients who did not obtain union had all received a marrow graft with <1000 progenitors/cm³ and <30,000 progenitors in total; both the mean concentration and the mean number were significantly lower than those for the patients for whom the treatment did not fail. Therefore, it seems reasonable to suggest that a graft needs to contain >1000 progenitors/cm³. This finding has implications regarding the intraoperative processing of bone marrow to select progenitors because bone marrow obtained by aspiration and not concentrated contains only a mean of approximately 600 progenitors/cm³ (range, 12 to 1224 progenitors/cm³).

Our results confirm that it is important to increase the number of progenitors in the graft after aspiration. Connolly et al.[7] examined the possibility of improving the efficacy of an aspirated bone-marrow graft by concentration in a study of animals. Even if the issue of concentration was not directly addressed by our experimental design, we were able to confirm its influence in our clinical study of humans by determining the number of cells in a standardized volume. However, it is not possible for us to know, from the findings in this study, whether the same number of cells in a smaller (or larger) volume would be similarly effective. The importance of the concentration of cells that can be delivered may be related to the survival of these progenitors after transplantation[20]. The amount of available oxygen is probably one of the limiting factors after transplantation. Since the transplanted progenitor cells compete with other cells for oxygen, one way to optimize cell survival is to limit the transplanted cells to those that contribute to the formation of bone (i.e., exclude all others). This was achieved by centrifugation in our series. Use of a porous implantable material has been reported as an alternative method for concentration and selection of connective-tissue progenitors[25]. Other methods to increase the population of progenitors in the bone marrow graft, such as the use of growth factors[26-29], will probably be proposed in the future.

Appendix

The quantity of medullary nuclear cells per kilogram of marrow was calculated with use of a formula that takes into account blood dilution. It was estimated that, in each milliliter of aspirate, medullary cells were represented by the difference between the nuclear cell count and the count in peripheral blood (sampled during the period of general anesthesia):

$$N(10^8/kg) = \frac{(V \times NP) - (V - 100) \times NS}{P}$$

where V = the total volume of aspirate in milliliters, including

the harvesting medium; NP = the nuclear cell count per milliliter in the collection bag, in which the harvesting medium is included, that leaves the operating room; V − 100 = the exact volume of aspirate, after subtraction of the 100 mL of harvesting medium; NS = the nuclear cell count per milliliter of peripheral blood drawn during the period of general anesthesia; and P = the patient's weight in kilograms. As an example: for a total final volume of 300 mL containing 14×10^6 nuclear cells/mL, obtained from a 70-kg adult with a leukocyte count of 4×10^6/mL as determined while the patient is under general anesthesia, it can be estimated that the medullary nuclear cell count is 5×10^7/kg, for a total of 0.35×10^{10} nuclear cells. ∎

Ph. Hernigou, MD
A. Poignard, MD

F. Beaujean, MD
H. Rouard, MD
Service de Chirurgie Orthopédique, Hôpital Henri Mondor, 94010 Creteil, France. E-mail address for Ph. Hernigou: philippe.hernigou@hmn.aphp.fr

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