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Existence of mesenchymal stem cells in sites of atrophic nonunion

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Objectives

Nonunion is one of the most troublesome complications to treat in orthopaedics. Former authors believed that atrophic nonunion occurred as a result of lack of mesenchymal stem cells (MSCs). We evaluated the number and viability of MSCs in site of atrophic nonunion compared with those in iliac crest.

Methods

We enrolled five patients with neglected atrophic nonunions of long bones confirmed by clinical examinations and plain radiographs into this study. As much as 10 ml bone marrow aspirate was obtained from both the nonunion site and the iliac crest and cultured for three weeks. Cell numbers were counted using a haemocytometer and vitality of the cells was determined by trypan blue staining. The cells were confirmed as MSCs by evaluating their expression marker (CD 105, CD 73, HLA-DR, CD 34, CD 45, CD 14, and CD 19). Cells number and viability were compared between the nonunion and iliac creat sites.

Results

After three weeks, numbers of 6.08×10^6 cells (SD 2.07) and 4.98×10^6 cells (SD 1.15) were obtained from the nonunion site and the iliac crest, respectively, with viability of 87.1% (81.7% to 90.8%) and 89.8% (84.7% to 94.5%), respectively. No differences was found between the two sources of MSCs regarding cells number ($p = 0.347$) and viability ($p = 0.175$).

Conclusions

Our findings showed the existence of MSCs in the site of atrophic nonunion, at a similar number and viability to those isolated from the iliac crest.

Keywords: Mesenchymal stem cells, Atrophic nonunion, Cell number, Cell viability, Iliac crest, Nonunion fracture

Article focus

- Evaluation of the number and viability of mesenchymal stem cells (MSCs) in sites of atrophic nonunion
- MSCs from bone marrow of nonunion site and iliac crest were aspirated and compared

Key messages

- Nonunion is one of the most troublesome complications to treat in orthopaedics
- Some authors believe that atrophic nonunion is as a result of poor vascularisation and/or lack of MSCs in the fracture site
- This study proves the existence of MSCs in sites of atrophic nonunion, and finds similar quantites and viability to the iliac crest

Strengths and limitations

- To our knowledge, we are the first to evaluate the existence of mesenchymal stem cells in the site of atrophic nonunion
- The limited number of subjects makes it difficult to draw definite conclusion
- We have not evaluated the differentiation capability of mesenchymal stem cells, and therefore further studies are needed

Introduction

Nonunion is one of the most difficult complications to treat in orthopaedics, often requiring re-admission and surgery.[1] It is also expensive, with an estimate of £13 844.68 required to treat one case of nonunion.[1] In Canada, it has been reported that treatment of a single case of nonunion can cost

between $6800 and $8200 USD.[2] Nonunion also decreases the function, productivity and quality of life of the patient.[3] If such a decrease in productivity is included in the estimation, the mean cost of treatment of a tibial nonunion can reach $18 712 USD.[3]

In order to treat cases of nonunion effectively, the surgeon must understand the underlying pathophysiology, which varies according to the type of nonunion.[4] In hypertrophic nonunion, callus forms and the fractures appear to have ubiquitous blood, oxygen and nutrient supply. The nonunion is therefore considered to be a result of insufficient stability and treatment is directed toward stabilisation of the fracture. However, the pathophysiology of atrophic nonunion, in which no callus is formed, is poorly understood. Poor vascularisation has been suggested as a cause of atrophic nonunion, with a study by Dickson et al[5] reporting that arterial occlusion in the ipsilateral extremity was associated with a higher rate of delayed union or nonunion in open fractures of the tibia. On the other hand, Brownlow, Reed and Simpson[6] found in an animal model that atrophic nonunions were well vascularised.

This observation was supported by Hernigou et al[7] who successfully treated 53 of 60 patients with atrophic nonunion of the tibia by percutaneous injection of concentrated bone marrow aspirate. It seemed that lack of osteoprogenitor cells was the cause of the atrophic nonunion. One may argue that the concentrated marrow used in Hernigou et al's[7] study contained not only osteoprogenitor cells but also growth factor for proliferation and differentiation of the osteoprogenitor cells, as well growth factor for neovascularisation. However, Centeno et al[8] have shown that percutaneous injection of autologous, culture-expanded, bone marrow-derived mesenchymal stem cells (MSCs) enhanced fracture healing in nonunion. It may be therefore that atrophic nonunion occurs as a result of a lack of MSCs at the site of nonunion, thus addition of MSCs alone was sufficient to promote bone healing in atrophic nonunion.[8] However, these acknowledged that platelet-derived growth factors may have played a role in their results.[8]

Whether poor vascularisation or lack of MSCs at the site of fracture is the primary cause of atrophic nonunion remains an unsolved controversy and warrants further study. In order to investigate the latter, we evaluated the number and viability of cultured MSCs from sites of atrophic nonunion and also from the iliac crest for comparison.

Materials and Methods

We enrolled five patients into this study with neglected atrophic nonunions of long bones confirmed by clinical examinations and plain radiographs. During routine open reduction and internal fixation for atrophic nonunion, and before recanalisation of the fracture, a 10 ml syringe prefilled with 2 ml heparin 1000 IU/ml was introduced until it reached the medullary canal. The plunger was then pulled to aspirate the marrow. The procedure was repeated until 10 ml of marrow was obtained. The surgery was then continued following the routine procedure. In addition, 10 ml of marrow was obtained from the iliac crest using the technique described by Lubis et al.[9]

In order to isolate and expand the MSCs, bone marrow aspirate from the atrophic nonunion site and iliac crest was diluted with an equal volume of phosphate buffered saline (PBS) and centrifuged at 3000 rpm for 30 minutes at room temperature. The buffy coat was re-suspended in low-glucose Dulbecco's modified Eagle's Medium (DMEM; Gibco, Grand Island, New York) and expanded in two 75 cm^2 tissue culture (TC) flasks. The suspension was incubated under 20% O_2 and 5% CO_2 at 37°C. At the end of the first week, the cells were washed with PBS and the medium was exchanged every two to three days for three weeks. The cells in the TC flasks were counted using a haemocytometer and their viability was determined by trypan blue staining. In order to ensure that the cultured cells were MSCs, we performed cell surface biomarker analysis by incubating cells with PE-conjugated mouse monoclonal anti-CD105 (Abcam, Cambridge, United Kingdom), PE-conjugated mouse monoclonal anti-human CD73 (BD Biosciences, San Jose, California), FITC-conjugated mouse monoclonal anti-human CD34 (BD Biosciences), FITC-conjugated mouse monoclonal anti-CD45 (BD Biosciences), FITC-conjugated mouse monoclonal anti-CD14 (Abcam), PE-conjugated mouse monoclonal anti-CD19 (Abcam) and PE-conjugated mouse monoclonal anti-HLA-DR+DP+DQ (Abcam) antibody. The expression marker was finally detected using FACSCalibur flow cytometer (Becton Dickson, San Jose, California).

Statistical analysis. The number and viability of MSCs were compared between the two sources of cells (site of atrophic nonunion and iliac crest) using the Mann-Whitney U test. A p-value < 0.05 was assumed to denote statistical significance. The statistical analyses were undertaken using SPSS v19 (SPSS Inc., Chicago, Illinois).

Results

The five patients were all male with a mean age of 27.4 years (18 to 37) at the time of the study. The site of fracture was the femur in three, the tibia in one and the humerus in one. The mean time since fracture (duration of nonunion) was 3.1 years (1 to 6) (Table I).

After three weeks, we obtained a mean of 6.08×10^6 cells (SD 2.07; 3.10 to 8.07) in the aspirates taken from the five sites of atrophic nonunion, compared with a mean of 4.98×10^6 cells (SD 1.15; 3.19 to 6.02) from the iliac crest aspirates (Table II). There was no statistically significant difference between the two sites (p = 0.347, Mann-Whitney U test). The mean viability of cells taken from sites of atrophic nonunion was 87.1% (81.7% to 90.8%) compared with 89.8% (84.7% to 94.5%) in samples from the iliac crest (Table II). Again, there was no statistically significant difference between the two sources of cells (p = 0.17, Mann-Whitney U test).

Table I. Characteristics of the patients

Patient	Age (yrs)	Gender	Fracture duration (yrs)	Site	Previous treatment	Comments
1	23	Male	6	Right femur	Splinting and massage by bone setter	Heavy smoker
2	37	Male	1	Right humerus	Splinting and massage by bone setter	Heavy smoker
3	33	Male	4	Right tibia	Splinting and massage by bone setter	History of open fracture
4	18	Male	3	Right femur	Splinting and massage by bone setter	-
5	26	Male	1.5	Right femur	1. Open reduction and internal fixation with plate and screws; 2. Second surgery for augmentation using hydroxyapatite–calcium sulphate synthetic bone graft	-

Table II. Cell number and viability after three weeks of culture in two tissue culture flasks and their expression marker

			Expression marker (%)						
Site of aspirate	Cells (n) ($\times 10^6$)	Viability (%)	CD105	CD73	HLA-DR	CD14	CD19	CD34	CD45
Patient 1									
Right femur	8.07	84.36	96.28	98.64	0.26	0.02	0.00	0.04	0.00
Iliac crest	5.34	84.72	99.52	99.34	0.04	0.00	0.00	0.00	0.00
Patient 2									
Right humerus	6.72	89.49	97.28	98.02	1.44	0.00	0.00	0.00	0.00
Iliac crest	6.02	86.32	96.82	96.56	0.56	0.00	0.00	0.00	0.00
Patient 3									
Right tibia	3.10	81.65	96.74	97.48	1.08	0.16	0.00	0.00	0.14
Iliac crest	3.19	91.25	97.42	96.5	1.96	0.26	0.00	0.00	0.00
Patient 4									
Right femur	7.65	90.80	98.10	99.48	0.54	0.10	0.00	0.00	0.00
Iliac crest	4.53	94.48	98.40	98.82	0.70	0.14	0.00	0.00	0.00
Patient 5									
Right femur	4.87	88.98	95.82	98.14	2.00	0.00	0.00	0.00	0.00
Iliac crest	5.80	92.19	99.52	99.34	0.04	0.00	0.00	0.00	0.00
Mean values (range)									
Nonunion site	6.08 (3.10 to 8.07)	87.06 (81.65 to 90.80)							
Iliac crest	4.98 (3.19 to 6.02)	89.79 (84.72 to 94.48)							
p-value*	0.347	0.170							

* Mann-Whitney U test

Discussion

The isolation of MSCs from normal femoral marrow has been investigated by Leonardi et al[10] and Ciapetti et al.[11] Leonardi et al[10] reported that femoral marrow was highly effective in proliferating and differentiating along the osteogenic lineage.[7] Ciapetti et al[11] found that MSCs isolated from the femur of adult patients consistently maintained an osteogenic potential. Our previous animal study also confirmed that marrow of long bone was an alternative source of plastic adherent cells to the iliac crest.[12] In the present study, we compared the cell number and viability between fracture site and iliac crest in the setting of atrophic nonunion.

We were able to isolate and expand MSCs from both the iliac crest and fracture site. MSCs were identified by the criteria advocated by the International Society for Cellular Therapy.[13] These criteria included adherence to plastic in standard culture conditions, positive expression of CD105, CD73, CD90 for at least 95%, negative expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR, as well as ability for *in vitro* differentiation into osteoblasts, adipocytes and chondroblasts.

We found comparable numbers and viability of MSCs at the site of fracture and at the iliac crest. The finding contradicts the belief that atrophic nonunion occurs as a result of lack of MSCs at the site of atrophic nonunion, and suggests that other pathophysiologies bear responsibility for the occurrence of atrophic nonunion. Possible pathophysiologies may include defective behaviour of the stem cells in their differentiation into osteogeneic cells. The ability to differentiate into the appropriate phenotype contributes substantially to the healing of fractures.[14]

Unfortunately, it was a limitation of our study that we did not evaluate the differentiation capability of the expanded stem cells. Moreover, our study included five subjects only, and the reults should therefore be interpreted with caution.

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- H. D. Ismail: Study concept, Performed surgeries, Data analysis, Critical review
- P. Phedy: Study concept, Assisted surgeries, Data collection, Data analysis, Writing the paper
- E. Kholinne: Study concept, Assisted surgeries, Data collection, Data analysis, Writing the paper
- Y. Kusnadi: Contribution to the research methods and techniques, Data collection, MSC processing and identification, Data analysis
- L. Sandhow: Contribution to the research methods and techniques, Data collection, MSC processing and identification, Data analysis
- M. Merlina: Contribution to the research methods and techniques, Data collection, MSC processing and identification, Data analysis

ICMJE Conflict of Interest:

- None declared