JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **16** (2005) 149–152

DBM induced ectopic bone formation in the rat: The importance of surface area

C. C. SCHOUTEN[1], ED H. M. HARTMAN[1,*], P. H. M. SPAUWEN[1], J. A. JANSEN[2]
Departments of [1]Plastic Surgery and [2]Biomaterials, University Medical Center Nijmegen, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands
E-mail: hartman@nvpc.nl

Demineralized bone matrix (DBM) has been shown to induce ectopic endochondral bone formation, when intramuscularly implanted in rats. In earlier studies we have found a variation in bone formation capacity of this DBM. This might be due to the properties of the DBM itself, but the use of DBM blocks could be of influence as well. Therefore, this study was designed to investigate whether increasing the surface area of the DBM by morsellizing, influences the bone formation capacity. In view of this, DBM implants and morsellized DBM (MDBM) implants were placed intramuscularly in a rat model. At six weeks the implants were retrieved and evaluated by histology and histomorphometry. The results demonstrated that significant amounts of newly formed bone were present in some DBM as well as some MDBM implants while in others no, or very little new bone was found. Histomorphometric analysis showed an average bone formation of 2.6% in DBM implants and an average of 1.9% in MDBM implants. Still, the amount of bone formation was limited compared with previous studies. It is concluded that enlargement of the surface area by morsellizing DBM implants is not an important factor in bone forming capacity.

1. Introduction

In reconstructive bone surgery donor site morbidity is a consequence of the use of autologous tissue [1]. Tissue engineering is a promising tool, which could help to minimise this donor site morbidity. One of the materials that has been studied extensively in bone regeneration procedures is demineralized bone matrix (DBM). As an osteoinductive agent, DBM induces osteogenesis via endochondral ossification [2–4]. The pioneering studies on bone induction were performed by Urist [5, 6]. He reported the isolation of a bone morphogenic protein (BMP) derived from bovine bone matrix gelatine by dissociative extraction in guanidine-HCl. This purified BMP, similar to DBM, was found to induce differentiation of mesenchymal cells into cartilage and bone when implanted into thigh-muscle pouches in mice. BMP has subsequently been isolated from rat, rabbit, guinea pig, porcine and human bone [4]. Glowacki and co-authors [7, 8] have recommended the following choices of DBM implant forms:

- powdered (75–250 um) for small irregular defects
- small chips (5 mm) for septic lesions
- corticocancellous blocks or strips for segmental defects

They caution that very large pieces of DBM should be avoided, since smaller ones have increased surface area, which ensures a greater accessibility to BMP [9]. This encourages the proliferation and differentiation of osteogenic cells and makes DBM granulate more osteoinductive [7, 8].

In earlier studies we have used blocks of DBM obtained from the femora of donor rats. These blocks were inserted a subcutaneously [2] as well as intramuscularly [3] in rats. Although significant bone formation occurred in both acceptor sites, we found a strong variation in bone forming capacity. This in contrast to Viljanen *et al*., who found almost complete ossification in their series of 12 DBM implants in a rat latissimus dorsi muscle flap model [10]. This difference in results can be due to the variation in bone forming capacity of the DBM itself, which has been described before [11]. It is possible that this effect was even more enhanced in our study, because we inserted the DBM as a block and not as a granulate.

On basis of the above mentioned, we decided to further explore the osteoinductive properties of DBM implants. We hypothesized that morsellized DBM (MDBM) has greater osteoinductive properties than non-morsellized DBM. Therefore, morsellized and non-morsellized DBM was inserted in a rat muscle

*Author to whom all correspondence should be addressed.

pouch. Bone formation was investigated by histology and histomorphometry.

2. Materials and methods

2.1. Implants

2.1.1. Demineralized bone matrix (DBM)

Ten 10-week male Wistar (W.U.) donor rats were sacrificed by an overdose CO_2. The femora of these donor rats were used to prepare 20 DBM-implants. Donor femora were cleaned of adherent periosteum, muscle and connective tissue. From the diaphysis of the resected femora, pieces with a length of 1.0 cm were cut and placed on ice, after flushing with saline to remove the bone marrow. The fragments were decalcified in 0.6 N HCl (1 g bone per 100 ml 0.6 N HCl) under constant stirring during 72 h at a temperature of 4 °C. After elution of the acid in 0.15 N NaCl, the fragments were lyophilised and stored at −20 °C. The average weight of the DBM was 3.1 (±0.4) mg.

2.1.2. Morsellized demineralized bone matrix (MDBM)

Ten pieces of the previously demineralized bone matrix were morsellized by mortar and pestle and bone chips were remodelled in a cylindrical mould by impaction. The average weight of the morsellized DBM implants was 2.8 (±0.5) mg.

3. Experimental design and surgical procedure

3.1. Surgery

For implantation ten 10-week old male Wistar acceptor rats (W.U., with an average weight of 280 g) were used. Surgery was performed under general inhalation anaesthesia using a mixture of fifty percent oxygen with fifty percent nitric oxide and 1.2% Isoflurane (Forene® (1-chloro-2, 2,2-trifluorethyl-difluoromethyl-ether). For the insertion of the implants, the animals were immobilised and placed in a supine position. Longitudinal skin incisions were made on the medial surface of both hind legs of the rat, from the ankle to the groin above the inguinal ligament. After the incision was made, an intramuscular pocket in the adductor thigh muscle was created by blunt dissection. The DBM implants were placed in the pocket, and the muscle and skin were closed using Agraven suture material. Each animal received one DBM implant as well as one morsellized DBM implant on the contralateral side. To assure statistical randomisation, DBM implants were alternately placed on the right or left side. In total 20 implants were placed. Six weeks after surgery the animals were sacrificed using an overdose of CO_2 anaesthesia.

The used animal protocol was approved by the Animal Care Ethics committee of the University of Nijmegen. The animal experiments were performed according guidelines for animal experiments for scientific research of the Dutch government.

3.2. Light microscopic evaluation

After sacrificing the animals, the implants with their surrounding tissue were retrieved and prepared for histological and histomorphometrical evaluation. For one implant histological and histomorphometrical evaluation was not possible as it was completely resorbed during the 6 weeks of implantation in the intramuscular pocket. All other implants were fixed in 4% buffered formaldehyde solution. Subsequently, the tissue blocks were dehydrated in ethanol and embedded in methylmethacrylate. After polymerisation, thin sections (thickness 10 μm) were made using a modified sawing microtome technique [12]. Transversal sections were made on 3 levels in each implant, with 3 slices per level. To be able to differentiate cartilage, bone, marrow and muscle the sections were stained with methylene blue (15 sec.) and basic fuchsin (30 sec.) and examined with a light microscope. To evaluate the tissue response to the implants, both histological and histomorphometrical evaluation was performed. The histological evaluation consisted of a complete description of the observed thin sections. For the histomorphometrical measurements image analysis techniques were performed. Therefore a computer based image analysis system (Leica Qwin) was used. The following parameters were assessed:

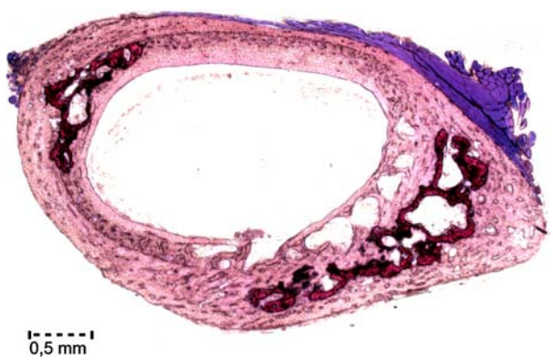
1. DBM resp. MDBM surface area
2. Surface area bone formation

All histomorphometrical measurements were performed in 9 different sections per implant. Presented results are based on the average of these measurements.

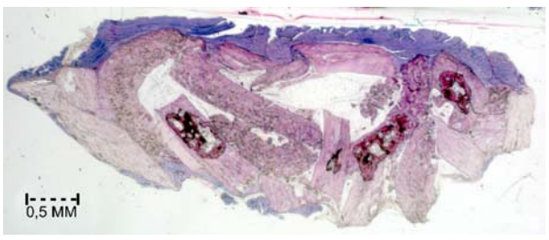
4. Results

4.1. Histological analysis

In DBM implants and morsellized DBM implants bone formation was seen (Fig. 1). However, a wide variation in the occurrence as well as amount of



(a)



(b)

Figures 1 (a) and (b). Bone formation at 6 weeks implantation in two implants, DBM (a) and MDBM (b) as studied by histology shows bone formation in both groups.

new bone formation was observed. In four out of nine DBM and one out of nine MDBM implants even no bone formation at all occurred. The deposited bone in the DBM implant had a trabecular appearance and could be associated with the presence of bone marrow-like tissue within the porosity of the bone trabeculae. The newly formed bone in the MDBM implants showed a more compact appearance than seen in the DBM implants. The bone was always characterised by the presence of osteocytes that were embedded in the mineralized matrix. At the outside, a periosteum-like tissue layer surrounded the bone. As shown in Fig. 1 the shape and size of the MDBM implants are different from the DBM implants. The natural oval-shape and collagenous structure of the DBM is hardly recognizable, as the MDBM implants have a more elongated form. Also, the location of the bone formation differed, i.e. in non-morsellized DBM distribution of bone formation was most pronounced at the distal part, while in morsellized DBM bone formation was most pronounced centrally. Almost no inflammatory cells were present close to the DBM or in between the MDBM particles, except for one DBM implant, where an inflammation was seen, which was associated with a lack of new bone formation.

4.2. Histomorphometrical evaluation

Results of histomorphometric analysis of the sections of both implants are shown in Fig. 2. In average 2.6% of bone formation was measured in DBM implants with a range from 0 to 14%. In MDBM implants, in average 1.9% bone formation was measured with a range from 0 to 9%.

5. Discussion

Demineralized bone matrix has been shown to induce ectopic endochondral bone formation, when intramuscularly implanted in rats. This study was designed to investigate whether increasing the surface area of DBM by morsellizing makes it more osteoinductive. This was done by comparing the osteoinductive properties of morsellized versus non-morsellized DBM by histology and histomorphometry. As described in previous reports, the induction of bone is related to the surface area of the exposed matrix. By morsellizing the DBM implant, the surface area increases and the implants'structure becomes less compact and therefore becomes more osteoinductive [7, 8]. Nevertheless, in this study we did not observe a difference between the amount of bone formation of DBM versus MDBM. Besides, we found that the new bone formation was very limited and showed a wide variance for both material preparations.

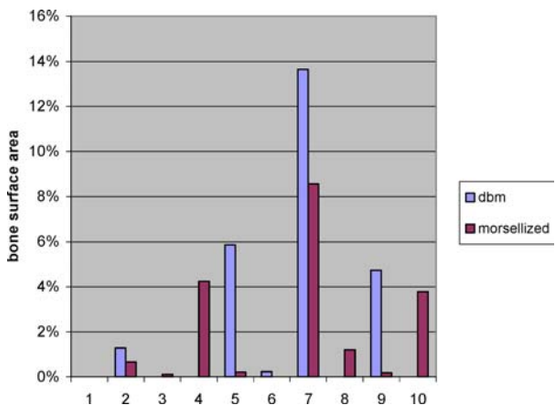


Figure 2 Histomorphometrical analysis of non-morsellized DBM and morsellized DBM showed limited bone formation for both groups. Non-morsellized DBM had an average bone formation of 2.6%, morsellized DBM 1.9%. There was no significant difference between these two groups.

Earlier performed studies [5, 8, 11, 13] showed general guidelines and test methods for assessment of the effectiveness of materials intended to induce bone formation when implanted in vivo. Following these guidelines we used in our experiment 10 week old rats of the male gender. Though gender is not a factor, the animals should be adolescent or young adults as older animals do not respond as effectively to osteoinductive materials [14]. Furthermore, we placed the implants in an intramuscular pocket in the adductor thigh muscle, as this site has proven to have greater potential for ectopic bone formation than subcutaneous tissue [15]. Also, we included on basis of previous studies, a sufficient number of samples per group in order to confirm the activity of DBM and to detect differences. Considering the fact that we met all these study design criteria, the following explanations can be given and remarks can be made dealing with our observations.

Firstly, the limited amount of new bone formation confirms again that demineralised bone is variable in its osteoinductive potential [10, 15, 19]. Of course, failure to detect reproducible bone induction using demineralized freeze-dried rat bone powder may be due to processing problems. For example, we know that the complete removal of all potentially inflammatory or antigenic substances is very important, since this can inhibit the osteoinductive properties of the DBM. Therefore, we followed a detailed procedure for the preparation of demineralized bone powder, which had been proven successful before [8]. In view of this, we have to notice that the inherent variability between donors and also unknown surgical as well as donor conditions can interfere with bone inductivity. In order to learn more about the importance and relevance of such difficult to control variables, it appears to be a prerequisite that in future studies controls with proven osteoinductive characteristics are included in the study design.

Secondly, comparison with our earlier subcutaneous DBM suggests that implant location is not a very relevant parameter for bone inductivity. This corroborates with one of our other studies in which we inserted titanium fiber mesh implants provided with bone marrow cells in the thigh muscle and subcutaneous tissue of rats [16]. Bone formation, as induced by the marrow cells, was observed in all titanium meshes, but the location of the construct did not have an effect at all on the initiation as well as amount of bone formation.

Finally, we have to notice that we cannot exclude that more bone formation occurred in our specimens

than determined in the histomorphometrical analysis. Histology is a destructive and time consuming technique. For example, only nine sections (at three different locations) could be analyzed in the current study. Bone formation in between the sections and selected areas will be missed and is impossible to avoid. Therefore, besides histology, other non-destructive methods, like micro-computer tomography (micro-CT), have to be recommended for the evaluation of bone formation in osteoinductivity studies.

6. Conclusion

Although in the literature it has been stated that the surface area of DBM is an important factor in the bone forming capacity of DBM, our results do not support this theory: enlargement of the surface area by morsellizing DBM implants did not lead to more bone formation. Significant amounts of newly formed bone were present in some DBM as well as MDBM implants while in others no or very little new bone was found. Still, the amount of bone formation was limited compared with previous studies. Therefore, in order to learn more and exclude unknown experimental variables, the inclusion of positive bone inductive controls as well the use of non-destructive analysis methods has to be recommended for future osteoinductivity studies.

References

1. E. H. HARTMAN, P. H. SPAUWEN and J. A. JANSEN, *J. Invest. Surg.* **15** (2002) 185.
2. E. H. HARTMAN, J. A. PIKKEMAAT, J. W. VEHOF, A. HEERSCHAP, J. A. JANSEN and P. H. SPAUWEN, *Tissue Eng.* **8** (2002) 1029.
3. E. H. M. HARTMAN, J. A. PIKKEMAAT, J. J. VAN ASTEN, *et al.*, *ibid.* 2004.
4. A. A. KALE and P. E. DI CESARE, *Am. J. Orthop.* **24** (1995) 752.
5. M. R. URIST, *Science* **150** (1965) 893.
6. M. R. URIST and A. J. MIKULSKI, *Proc. Soc. Exp. Biol. Med.* **162** (1979) 48.
7. J. GLOWACKI, L. B. KABAN, J. E. MURRAY, J. FOLKMAN and J. B. MULLIKEN, *Lancet* **1** (1981) 959.
8. J. GLOWACKI and J. B. MULLIKEN, *Clin. Plast. Surg.* **12** (1985) 233.
9. J. J. WU and D. R. EYRE, *Calcif. Tissue. Int.* **42** (1988) 243.
10. V. V. VILJANEN, T. J. GAO and T. S. LINDHOLM, *J. Reconstr. Microsurg.* **13** (1997) 207.
11. Z. SCHWARTZ, J. T. MELLONIG, D. L. CARNES JR., *et al.*, *J. Periodontol.* **67** (1996) 918.
12. H. B. VAN DER LUBBE, C. P. KLEIN and K. DE GROOT, *Stain. Technol.* **63** (1988) 171.
13. M. R. URIST, J. J. CHANG, A. LIETZE, Y. K. HUO, A. G. BROWNELL and R. J. DELANGE, *Methods Enzymol.* **146** (1987) 294.
14. S. K. NISHIMOTO, C. H. CHANG, E. GENDLER, W. F. STRYKER and M. E. NIMNI, *Calcif. Tissue. Int.* **37** (1985) 617.
15. R. K. KHOURI, D. M. BROWN, B. KOUDSI, *et al.*, *Plast. Reconstr. Surg.* **98** (1996) 103.
16. E. H. M. HARTMAN, J. W. M. VEHOF, J. E. DE RUITER, P. H. M. SPAUWEN and J. A. JANSEN, *Biomaterials* 2004.

Received 27 April
and accepted 19 July 2004