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THE OSTEOGENIC POTENTIAL OF FRACTURE HEMATOMA AND ITS MECHANISM ON BONE FORMATION
— THROUGH FRACTURE HEMATOMA CULTURE AND TRANSPLANTATION OF FREEZE-DRIED HEMATOMA —

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INDEXING WORDS

fracture hematoma, culture, freeze-dried hematoma

SYNOPSIS

This study was conducted to provide further evidence of the inherent osteogenic potential of the fracture hematoma. The fracture hematoma was separated into its cellular and extracellular elements. The hematoma cells were cultured to study bone formation by the cellular elements alone. Bone formation acceleration factor was added to the cultured fracture hematoma cells. The cell responded to this stimulation by differentiation into chondrocytes.

Fracture hematoma was freeze-dried to study the presence of osteoinduction by the extracellular factors in the fracture hematoma. The freeze-dried fracture hematoma was packaged in methylmethacrylate pellets and within capsules of hydroxyapatite. These pellets and capsules in response to extracellular humoral factors from the freeze-dried fracture hematoma.

The results of experimental implantation of the cultured fracture hematoma cells revealed that these cells had the potential to differentiate into chondroblasts or osteoblasts when bone induction factors and bone acceleration factor was added to their media. These

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extracellular humoral factors are known to be present in the fracture hematoma.

INTRODUCTION

The precise role of fracture hematoma in fracture healing is not completely understood. It has been reported that the fracture hematoma does nothing more than span the gap between the ends of the fractured bone (2). In other studies the hematoma has been found to function as an important scaffold linking the ends of the fractured bone (3). The organized fracture hematoma has been identified as a framework for the later development of fracture callus (8). Yet, there is also literature which maintains that the fracture callus is unnecessary for fracture healing (11,13).

Mizuno, et al has published research in which fracture hematoma was implanted to a subperiosteal site at the parietal bone and to an intramuscular site in rats (6,7). They reported that the implanted hematoma had inherent osteogenic potential because it resulted in new bone formation at both the subperiosteal and intramuscular sites.

The purpose of this study was to investigate the source of the demonstrated osteogenic potential of the fracture hematoma. The authors hypothesized that the osteogenic potential of the fracture hematoma may arise from either the cellular or extra - cellular elements of the hematoma. This paper will describe the authous investigation into the osteogenic poptential of the cellular and extracellular components of the fracture hematoma.

The osteogenic potential of the extracellular elements of the fracture hematoma was investigated with a freeze-dried pellet of fracture hematoma. It was implanted into different sites which were later examined for new bone formation.

The osteogenic potential of the cellular elements of the fracture hematoma was investigated with the use of cell culture techniques. Fresh fracture hematoma was placed into cell culture. The tissue grown in cell culture from the fracture hematoma was then implanted into different sites which were later examined for new bone formation.

METHOD

Experiment 1; Study of the Extracelluar Element

1) Preparation of the Freeze-dried Fracture Hematoma

A closed fracture was manually performed on the femur of an anesthetized rat. Four days after the fracture, the fractured site was surgically exposed and the fracture hematoma was harvested. The fracture hematoma was frozen at -70°C. It was then vacuum freeze-dried at -80°C for 48 hours. The freeze-dried hematoma was then used for experimental implantation.

2) Experimental Implantation of the Freeze-dried Hematoma

A. The preparation of the freeze-dried hematoma pellet.

The fracture hematoma immediately after freeze-drying existed as a powder which was not suitable for implantation. To form a pellet which was easily implanted the freeze-dried fracture hematoma was enclosed within a methylmethacrylate polymer. Forty-eight Wistar rats which had been anaesthetized with nembutol had the freeze-dried fracture hematoma pellet implanted to a subperiosteal location at the parietal bone in twenty-four rats, and to an intramuscular site at the back in other twenty-four rats. After implantation of the hematoma the rats were returned to their cages without immobilization or restriction of activity. The rats were sacrificed at 2 weeks, 3 weeks, and 4 weeks after implantation.

At the time of sacrifice the implanted pellet and the surrounding tissue was removed en bloc. The specimen containing the freeze-dried fracture hematoma pellet and surrounding tissue was first examined with soft X-rays. The specimen was then decalcified for histological investigation. The histological specimens were stained with hematoxylin and eosin, toluidine-blue, and examined with routine light microscopy.

Controls for this experiment were produced with the implantation of freeze-dried peripheral blood hematoma in eight rats, and the implantation of an empty methylmethacrylate polymer pellet in eight rats. These two implants were harvested, examined and processed in an identical fashion to the pellets which contained the freeze-dried fracture hematoma.

B. Preparation of a hydroxyapatite capsule

A hydroxyapatite capsule was selected as an additional implant in

which powdered freeze-dried fracture hematoma could be experimentally implanted into rats. It was hypothesized that hydroxyapatite may be more suitable for new bone formation than methylmethacrylate.

The hydroxyapatite capsule was the shape of a cylinder, its diameter was 6.7 mm. The inside diameter was 5.8 mm, and pore diameter was 200 micro meter. The porosity was 70 ± 3 % and the calcinatory temperature was 900°C. The freeze-dried fracture hematoma was placed within the hydroxyapatite capsule. As a rat was too small to insert this capsule into the bone, a rabbit was used in this experiment.

The hydroxyapatite capsule containing the freeze-dried fracture hematoma was implanted into the tibial condyle of eight rabbits.A hole was drilled into the condyle with the same diameter of the capsule. The capsule was then inserted into this hole with manual pressure. Four weeks after implantation of the capsule,the rabbit was sacrificed and entire tibial condyle containing the capsule was harvested, examined with soft X-ray, and then decalcified for histological examination. A control for this experiment was performed with the identical implantation and examination of an empty hydroxyapatite capsule in eight rabbits.

Experiment 2; The Cellular Element of the Fracture Hematoma

1) The Culture of Fracture Hematoma Cells

A closed fracture was manually performed on the femur of an anaesthetized rats. Four days after the fracture, the fracture site was surgically exposed and the fracture hematoma was harvested. The fracture hematoma was cultured by RPMI-1640 including 10% fetal bovine serum (FBS). Numerous growth stimulating agents were added to the culture media while the fracture hematoma cells were incubating. These agents included; 1. Transforming growth factor beta (TGF-β), 2. Endothelial cell growth Factor (ECGF), 3. Interleukin-1 (IL-1). These three growth stimulating agents were added to the fracture hematoma cell cultures at four different concentrations. These concentrations were; 0.1 ng/ml, 1.0 ng/ml, 10.0 ng/ml, 100 ng/ml. Forty-eight hours after initiation of the cell culture, DNA synthesis of the hematoma cells was confirmed using ^{3}H-thymidine labeling. The growth stimulating cytokines were added to the cell cultures for one week, thereafter the cell cultures were examined to assess the degree of cellular growth.

2) Implantaion of the Cultured Cells of the Hematoma

Sixteen Wistar rats which had been anaesthetized with nembutol had the cultured cells of the fracture hematoma implanted to a subperiosteal location at the parietal bone. These cells had been in cell culture for one week. In this portion of the experiment, two identical groups of rats were established. One greoup of eight rats received hematoma cell cultures which had been stimulated with growth factors. In the second, control group of eight rats, the hematoma cells had received no stimulating factors prior to implantation. The harvested specimens were decalcified and examined with standard light microscopy for new bone formation.

RESULTS

1. Implantation of the Freeze-dried Hematoma

1) Freeze-Dried Hematoma with Methylmethacrylate Pellet

The freeze-dried hematoma powder had been combined with methylmethacrylate to form an implantable pellet. These pellets had been implanted to a subperiosteal location at the parietal bone. Histological examination of specimens which had been harvested at two weeks after implantation showed inflammatory reaction and new vcascularization at the boundry of the pellet and the bone. New bone formation was observed arising from the elevated periosteum of the parietal bone in six of eight rats (Fig. 1). This new bone was advancing toward the pellet, but had arisen from the parietal bone, not the pellet. No new bone formation was observed from the fracture hematoma pellet.

At four weeks after implantation, new bone was observed arising from the elevated periosteum and the parietal bone. This new bone had advanced to the boundry between the implanted pellet and the parietal bone in five of eight rats, but bone did not appear to have arisen from the pellet. This new bone was undergoing resorption and inflammatory reaction was already subsiding.

The control pellets containing freeze-dried peripheral blood hematoma or no hematoma, showed the elevated periosteum of the parietal bone. But the control pellets did not stimulate bone formation or a foreign body reaction at two and four weeks after implantation. Multinuclear giant cells were not observed.

The freeze-dried hematoma powder had been combined with methylmethacrylate to form an implantable pellet which had then implanted to an intramuscular location at the back. Histological

evaluation of these pellets and their surrounding tissues showed a mild inflammatory reaction. However, no new bone was observed arising from the pellet with fracture hematoma or from the control pellets.

2) Freeze-Dried Hematoma in Hydroxyapatite Capsule

In the animals in which hydroxyapatite capsules containing the freeze-dried fracture hematoma were implanted, abundant new bone formation was observed inside the pores of the hydroxyapatite in all rabbits. New bones had advanced to the freeze-dried hematoma (Fig. 2-A). However, these new bones did not arise from the implanted freeze-dried fracture hematoma. In the implanted empty hydroxyapatite capsules identical observations were made inside the pores, but advancement of new bone to the capsular cavity was not seen (Fig. 2-B). Abundant new bone formation was seen with respect to the pores of the hydroxyapatite capsule, but no new bone was observed at the interface between the capsule and bone.

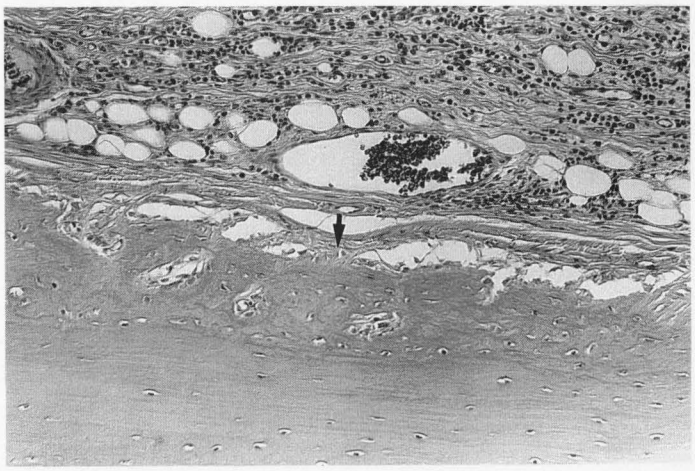


Fig. 1
Histological findings at two weeks after implantation of the freeze-dried hematoma with methylmethacrylate pellet. New bone formation (arrow) can be seen arising from the elevated periosteum of the parietal bone. (Hematoxylin - Eosin staining X 200).

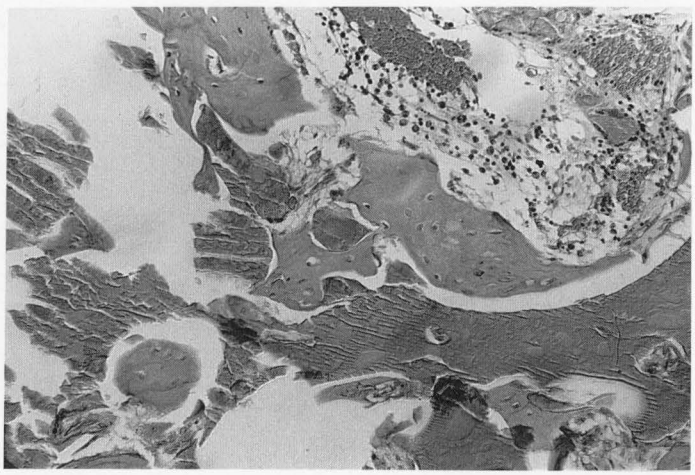
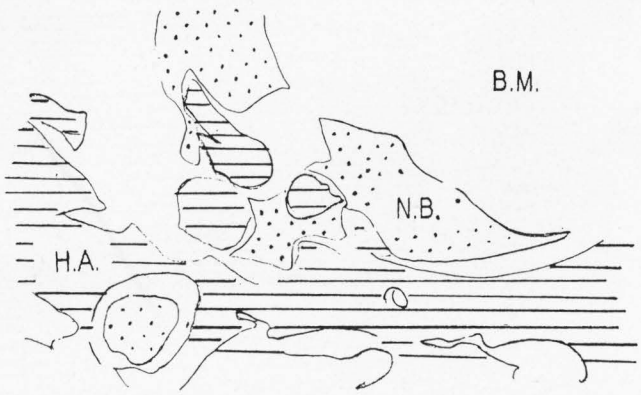


Fig. 2A
Histological findings at four weeks after implantation of the freeze-dried hematoma with the hydroxyapatite capsule. Abundant new bone formation can be seen inside the pores of the hydroxyapatite. New bones are advancing to the freeze-dried hematoma. (H - E staining X200)



B. M. : Bone Marrow H. A. : Hydroxyapatite N. B. : New Bone

Fig. 2A'

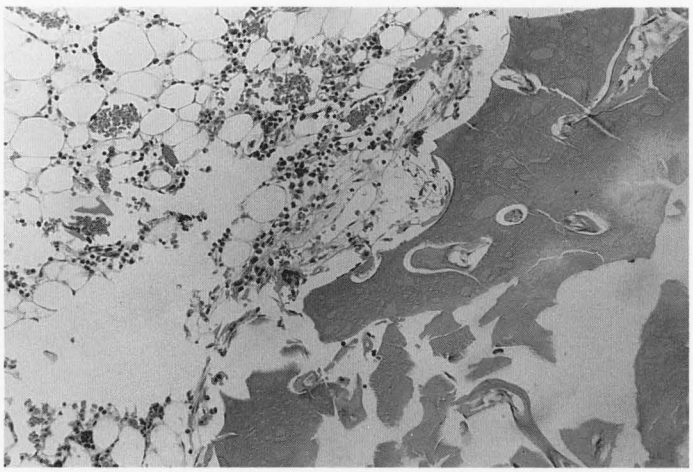
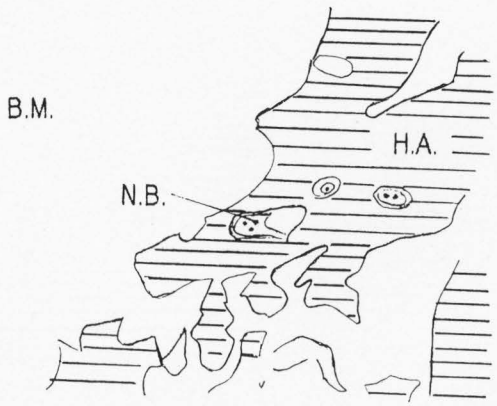


Fig. 2B
Histological findings of the control at four weeks after implantation of the empty hydroxyapatite capsules without the freeze-dried hematoma.
New bone formation can be made inside the pores, but advancement of new bone to the capsular cavity can not be seen (H - E staining X200).



B. M. : Bone Marrow H. A. : Hydroxyapatite N. B. : New Bone

Fig. 2B'

2. The Culture of Fracture Hematoma Cells and their Implantation

1) The Culture of the Fracture Hematoma

Four days after fracture, mesenchymal cells with primarily young osteoblasts, were the predominant cell lines in the harvested fracture hematoma (Fig. 3). These cells from the fracture hematoma were then nurtured in cell culture. The appearance of these cells resembled spindles and fibroblast-like cells. The bimorphic shape of the cells was preserved even though the second generation of the cells (Fig. 4).

Cytokines were added to each of the cell cultures to promote cell growth. Three cytokines were individually added to the cell cultures, and each cytokine was added in four levels of concentration. The three cytokines which were added to the cultures were TGF-β, ECGF, and IL-1. The different concentrations of the individual cytokines showed no apparent difference on the incorpolation of ^{3}H-thymidine.

The cytokine TGF-β had an apparent effect on the cell cultures. Cell cultures which had received this cytokine had a different appearance from those which had not (Fig. 5). Toluidine-blue darkly stained both the cellular and extracellular elements in cell cultures which had received TGF-β. This significant staining strongly suggested the presence of cartilage matrix. This type of staining was not observed in the cell cultures which had received the other cytokines.

2) Transplantation of the Cultured Cells

The cells from the cultures which had been stimulated by the TGF-β were implanted to subperiosteal location at the parietal bone in four of eight rats. Two weeks after implantaion, chondrocytes were observed at the site of implantaion (Fig. 6-A). In contrast, no chondrycytes were observed at the sites where cultured cells that had not received TGF-β had been implanted in eight rats (Fig. 6-B).

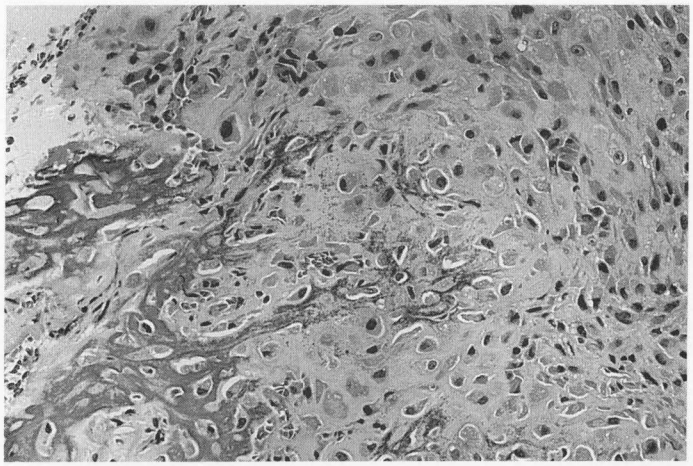


Fig. 3
Histological findings of the fracture hematoma harvested four days after fracture. Mesenchymal cells with primarily young osteoblasts are the predominant cell lines in the harvested fracture hematoma (H - E staining X200)

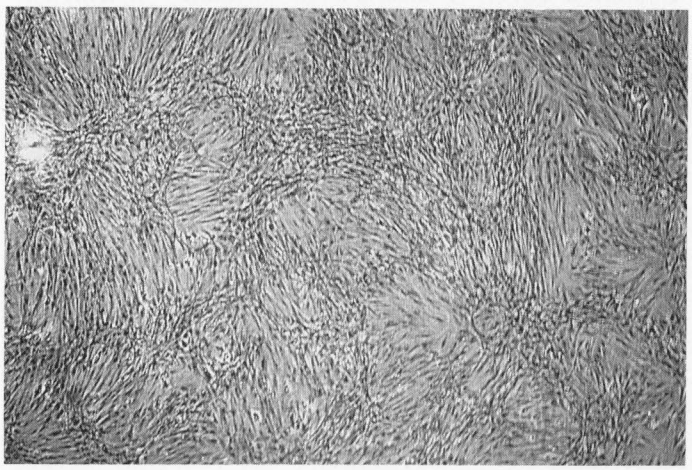


Fig. 4
Appearance of the cultured cells of the fracture hematoma through the second generation. The biomorphic shape of the cells is preserved. (X40)

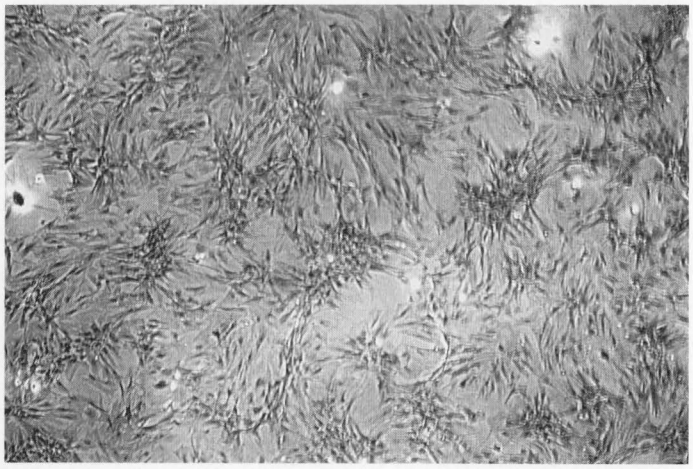


Fig. 5
Appearance of the cultured cells of the fracture hematoma, which were added the cytokine TGF-β. Cell cultures had a different appearance from those which had not. Toluidine-blue darkly stained both the cellular and extracellular elements in cell cultures. (X40)

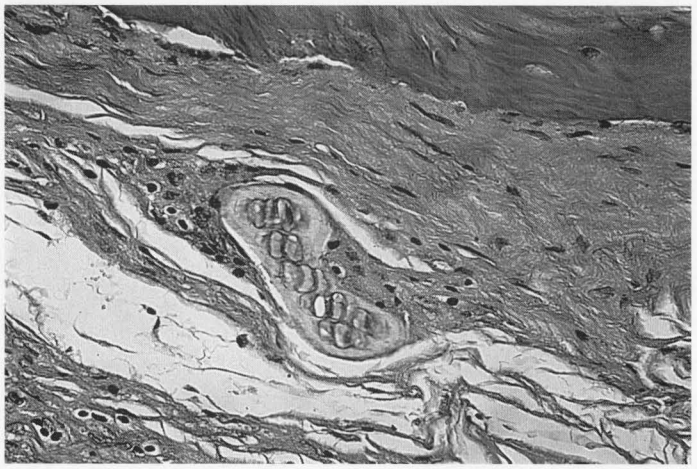


Fig. 6 A
Histological findings at two weeks after implantation of the cultured cells, which had received the cytokine TGF-β, to a subperiosteal location at the parietal bone. Chondrocytes can be seen at the site of implantation. (H - E staining X200)

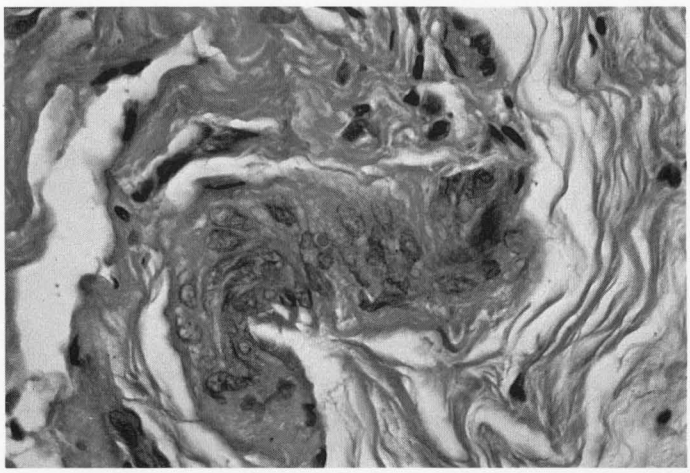


Fig. 6B
Histological findings at two weeks after implantation of the cultured cells, which had not received the cytokine TGF-β, to a subperiosteal location at the parietal bone. Chondroxcytes can not be seen at the site of implantation. (H - E staining X200)

DISCUSSION

It has been described and supported by research that the fracture hematoma is important for fracture healing. The exact role of the fracture hematoma has not been completely defined. Brighton has described the fracture hematoma as a biological bridge to span the fracture gap (2). Cruess, et al has described it as the framework for callus formation at the fracture site (3). Anders, et al has reported that the fracture site has the ability to produce reparative bone (1). Mizuno, et al has reported that the fracture hematoma has an inherent osteogenic potential which significantly contributes to fracture healing (6,7). Therefore, recent literature has supported the fracture hematoma has important properties for fracture healing, but the mechanism of the osteogenic potenital of the fracture hematoma is not understood.

There have been many papers which have reported on the mechanisms of bone formation. The osteoinductive and osteoconductive properties of many tissues and substances have been explored. It is well known

that decalcified bone, and decalcified bone with bone marrow can be osteoconductive and osteoinductive (4,9,12,14,15,17).

Certain cytokines have been identified which have a significant role in stimulating new bone formation. Implantation of bone morphogenic protein (BMP), to a subfascial or intra-abdominal location induced new bone formation at these sites (15,16). Continual infusion of TGF-β to a subperiosteal location at the parietal bone also stimulated new bone formation (5,10).

The mechanism by which cytokines stimulate new bone formation has been through the stimulation of undifferentiated mesenchymal cells which are abundant on bone surfaces. The cytokines stimulate these primordial cells to differentiate and produce bone. The transformation of undifferentiated cells towards cells with specific bone producing capabilities may be stimulated by the cytokines and BMP, such as TGF-β or fibroblast growth factor (FGF).

The authros, and previous reports by Mizuno have described the osteogenic potential of the fracture hematoma (7). However, the mechanims of the osteogenic potential of the fracture hematoma is not udnerstood and requires further investigation. The authors planned this study with two accepted assumptions that new bone formation requires two factors. The first factor is cellular. New bone cannot form without the appropriate precursor cells to differentiate into osteoblasts. The second factor is humoral. There must be an extracellular bone induction factor or factors which can induce undifferentiated cells to the fracture site and stimulate their maturation into ostaoblasts.

It is reasonable that the fracture hematoma would contain both the humoral and cellular factors to initiate fracture healing and new bone formation. The authors sought to separate these two factors of new bone formation by isolating the cellular elements in cell cuture, and the humoral factors with freeze-drying.

The result of the implantation of the freeze-dried hematoma showed that the freeze-dried hematoma did not convert to bone, and had no osteoconductive function. Yet, new bone was observed in its direct proximity which would suggest that the humoral factors of the freeze-dried hematoma had demonstrated osteoinductive ability.

The cellular element of the fracture hematoma, nurtured in cell culture, clearly showed the ability to differentiate into bone and cartilage. The authors have made four observations which support their

conclusion that the cellular element has the potential to produce cartilage and bone. First, the cellular elements harvested from the fracture hematoma were morphologically undifferentiated mesencymal cells which resembled fibroblasts. Second, the cell culture in the second generation of cells continued to show a powerful vital force. Third, the cultured cells produced cartilage matrix when stimulated by TGF-β. Fourth, when the cultured cells were implanted into the organism they proceeded to become chondrocytes. It is generally impossible to prove the process of cellular differentiation from chondrogenesis through bone formation in vitro.

Examination of the observations in this study suggest that the fracture hematoma has both an osteoinductive factor and direct cellular osteogenic factor. In the healing process of fracture, the fracture hematoma is not only a passive bridging structure. The fracture hematoma also has a significant dynamic role in fracture healing.

The fracture hematoma contains humoral elemlents which are osteoinductive. While this research has shown that these elements have a significant effect on stimulating new bone formaiton, in this research the humoral elements of the freeze-dried fracture hematoma were used collectively and not individually separated. Further research is requried to isolate the different cytokines, bone induction factors, bone acceleration factors or other factors which are present in the fracture hematoma.

ACKNOWLEDGEMENT

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