

**DTIC FILE COPY**

2

FINAL REPORT FOR

**"Healing of Intraosseous Critical Size Defects in Long-Evans Rats"**

Leslie J. Marden  
Patrick Canan  
Nicholas Quigley  
A. Hari Reddi  
Jeffrey O. Hollinger

**DTIC**  
SELECTE  
MAR 26 1991

**NOTEBOOK SERIAL #:** 401

**PROTOCOL #:** 245

**ANIMAL PROTOCOL #:** D02-90



UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED		1b. RESTRICTIVE MARKINGS			
2a. SECURITY CLASSIFICATION AUTHORITY UNCLASSIFIED		3. DISTRIBUTION/AVAILABILITY OF REPORT This document has been approved for public release; its distribution is unlimited.			
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE					
4. PERFORMING ORGANIZATION REPORT NUMBER(S)		5. MONITORING ORGANIZATION REPORT NUMBER(S)			
6a. NAME OF PERFORMING ORGANIZATION US Army Institute of Dental Research		6b. OFFICE SYMBOL (If applicable) SGRD-UDR-S	7a. NAME OF MONITORING ORGANIZATION US Army Medical Research & Development Command (HQDA-IS)		
6c. ADDRESS (City, State, and ZIP Code) Walter Reed Army Medical Center Washington, DC 20307-5300		7b. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, MD 21702-5014			
8a. NAME OF FUNDING/SPONSORING ORGANIZATION		8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER		
8c. ADDRESS (City, State, and ZIP Code)		10. SOURCE OF FUNDING NUMBERS			
		PROGRAM ELEMENT NO. 060287A	PROJECT NO. 3M162787A825	TASK NO. ED	WORK UNIT ACCESSION NO.
11. TITLE (Include Security Classification) Healing Of Intraosseous Critical Size Defects In Long-Evans Rats					
12. PERSONAL AUTHOR(S) Leslie J. Marde; Patrick Canan, Nicholas Quigley, A. Hari Reddi, Jeffrey O. Hollinger					
13a. TYPE OF REPORT Final		13b. TIME COVERED FROM Oct 89 TO Dec 90	14. DATE OF REPORT (Year, Month, Day) 91/01/10		15. PAGE COUNT 10
16. SUPPLEMENTARY NOTATION					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP	Craniotomy defects(CSDs),osteoconductive, osteoinductive		
06	04				
19. ABSTRACT (Continue on reverse if necessary and identify by block number) Repair of rat critical-size craniotomy defects (CSDs) was compared with that of CSDs treated with either osteoconductive rat insoluble collagenous bone matrix (M), or osteoinductive partially purified bovine osteogenin, reconstituted with M (OG/M). Repair of all CSDs was similar histologically throughout the first three days, characterized by acute, then chronic inflammation and granulation tissue formation. Osteoclastic and osteoblastic activity at the margins of the defects subsided by day 28 in untreated and M-treated defects. Untreated CSDs gradually filled in with fibrous connective tissue which matured throughout 42 days. CSDs treated with M showed island of cartilage and bone embedded in connective tissue at day 9, which reached peak maturity by day 14. In contrast, cartilage and osteoblast were present in defects treated with OG/M at day 5. By day 9 cartilage and osteoid production were active. New bone formed at the edges of the defects showed hematopoietic tissue by day 11; a complete bone bridge was established by day 21. By day 42, fatty marrow was present. Radiopacity, quantified and computerized image analysis, increase dramatically between days 9 and 14 in					
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION Unclassified		
22a. NAME OF RESPONSIBLE INDIVIDUAL Jean A. Setterstrom		22b. TELEPHONE (Include Area Code) 202-576-3484		22c. OFFICE SYMBOL SGRD-UDR	

19. ABSTRACT (CONTIUNED)

OG/M-treated defects, and remained greater ( $p < 0.05$ ) than that of the M-treated group throughout day 42. There was a more gradual increase in radio-pacity in M-treated defects; by day 72 there was no difference between defects treated with OG/M and M. The chronology of ortho-topic bone formation in CSDs was very similar to that for OG/M-induced heterotopic bone formation.

## ABSTRACT

Repair of rat critical-size craniotomy defects (CSDs) was compared with that of CSDs treated with either osteoconductive rat insoluble collagenous bone matrix (M), or osteoinductive partially purified bovine osteogenin, reconstituted with M (OG/M). Repair of all CSDs was similar histologically throughout the first three days, characterized by acute, then chronic inflammation and granulation tissue formation. Osteoclastic and osteoblastic activity at the margins of the defects subsided by day 28 in untreated and M-treated defects. Untreated CSDs gradually filled in with fibrous connective tissue which matured throughout 42 days. CSDs treated with M showed islands of cartilage and bone embedded in connective tissue at day 9, which reached peak maturity by day 14. In contrast, cartilage and osteoblasts were present in defects treated with OG/M at day 5. By day 9 cartilage and osteoid production were active. New bone formed at the edges of the defects showed hematopoietic tissue by day 11; a complete bone bridge was established by day 21. By day 42, fatty marrow was present. Radiopacity, quantified by computerized image analysis, increased dramatically between days 9 and 14 in OG/M-treated defects, and remained greater ( $p < 0.05$ ) than that of the M-treated group throughout day 42. There was a more gradual increase in radiopacity in M-treated defects; by day 72 there was no difference between defects treated with OG/M and M. The chronology of orthotopic bone formation in CSDs was very similar to that for OG/M-induced heterotopic bone formation.



UNCLASSIFIED		<input checked="" type="checkbox"/>
UNRECORDED		<input type="checkbox"/>
Justification		
By _____		
Distribution/		
Availability Codes		
Avail num/or		
Dist	Special	
A-1		

## **INTRODUCTION**

Osteogenin (OG) is a glycoprotein which induces bone formation (Sampath et al. 1987). When reconstituted with noninductive, insoluble collagenous bone matrix (ICBM) and implanted in extrasketal sites in rats, an ossicle of bone forms within 28 days. The molecular mechanism of this induction is not yet known. Treatment of 8-mm circular craniotomy defects in rats with OG/ICBM induces bone regeneration (Mark et al. 1990). If left untreated, this defect would not heal by bone formation within the lifetime of the rat (Takagi and Urist 1982).

We wanted to identify the cellular events occurring during orthotopic bone regeneration and compare them to those observed during heterotopic bone induction (Reddi 1985).

## **METHODS**

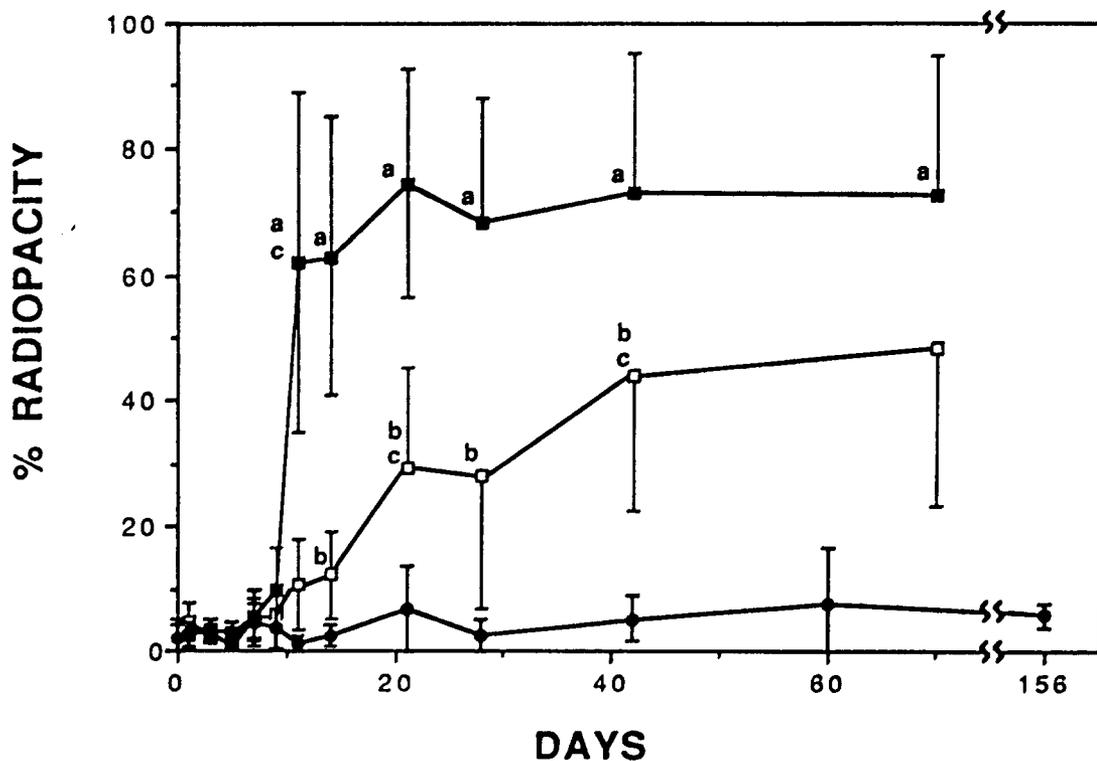
**Materials:** Insoluble collagenous bone matrix was prepared by extracting demineralized bone matrix powder with 4 M guanidine. The residue was washed extensively with water and lyophilized. OG was partially purified from bovine long bone by heparin sepharose, hydroxyapatite, and size exclusion column chromatography (Luyten et al. 1989).

**Methods:** 8-mm implants were prepared by sedimentation of 150  $\mu$ g OG, reconstituted with 25 mg ICBM powder by ethanol precipitation. Craniotomy defects were treated with ICBM alone, OG/ICBM, or left untreated. At discrete times, recipient beds from ten rats within each treatment group were retrieved, radiographed, and embedded in polymethylmethacrylate. 4.5-5.0  $\mu$  frontal sections, stained with von Kossa or Goldner's trichrome stains were examined subjectively. Radiopacity and new bone calcium were quantified by computerized image analysis.

## RESULTS

Subjective analysis of histologic sections of the craniotomy defect sites is summarized in Table 1 and is compared with events which occur during heterotopic bone induction (Reddi 1985).

Radiomorphometric analysis (Figure 1.) showed very sparse radiopaque material inside the 8-mm defect. There was some bone regeneration in defects treated with insoluble matrix alone. However, treatment of the defect with OG resulted in a dramatic increase in radiopaque material within the wound at 11 days, and which was sustained throughout the 70-day period.



- a = significantly different from matrix-treated ( $p < 0.05$ )
- b = significantly different from untreated ( $p < 0.05$ )
- c = significantly different from previous time point ( $p < 0.05$ )

**Figure 1. Changes in radiopacity within 8-mm critical-size defects with time.** Defects were left untreated ●, or were treated with 25 mg insoluble collagenous bone matrix (ICBM) □, or 150  $\mu$ g osteogenin reconstituted with 25 mg ICBM ■. Error bars represent one standard deviation.

**Table 1. Histologic changes in 8-mm craniotomy defects in response to no treatment, treatment with insoluble collagenous bone matrix (ICBM), or osteogenin plus ICBM, compared with changes in response to implantation of OG/ICBM heterotopically (Reddi 1985).**

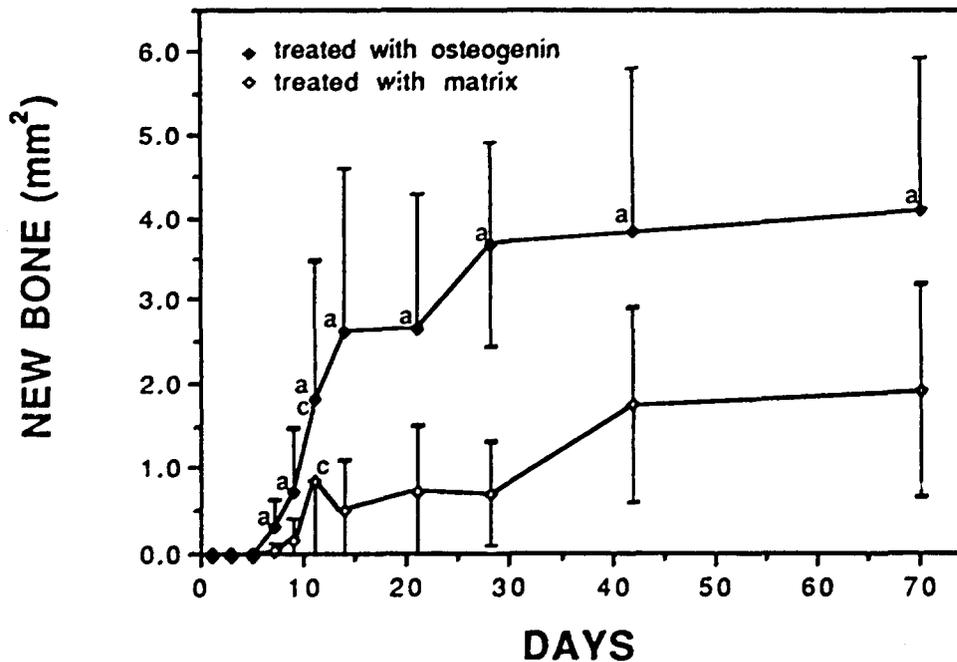
DAY	HETEROTOPIC OG/ICBM	UNTREATED CRANIOTOMY	CRANIOTOMY TREATED WITH ICBM	CRANIOTOMY TREATED WITH OG/ICBM
30 min		Superficial fibrin clot of varying thickness. Vascular dilation. Exudate.		
60 min		Fibrin clot still present. Pyknosis of osteocytes adjacent to surgical margin of bone.		
1	PMNs. Fibrin.	PMNs. Fibrin clot still present. Edema and brain necrosis. Pyknotic osteocytes at wound margin. Plump mesenchymal cells in connective tissue between brain and wound.	Acute inflammatory reaction, generally mild to moderate, occasionally heavy. ICBM surrounded by edema.	Mild to heavy inflammatory response, predominately PMNs around ICBM. Particles interspersed with edema fluid and fibrin.
3	Fewer PMNs. Mesenchymal cells in contact with ICBM.	Granulation tissue organized at periphery of wound. Decreased inflammation. Dead bone fragments and debris associated with macrophages and few multinucleated giant cells.	Granulation tissue organized at periphery of wound, beginning to surround ICBM. Decreased inflammation.	Granulation tissue. Plumping of stromal cells. Prevalent mitoses. New vessels at edges of defect. Less inflammation at the surgical edges, chronic inflammatory cells. ICBM in central portion of defect surrounded by edema, mostly acute inflammation, fibrin.

DAY HETEROTOPIC OG/ICBM	UNTREATED CRANIOTOMY	CRANIOTOMY TREATED WITH ICBM	CRANIOTOMY TREATED WITH OG/ICBM
5	<p>Granulation tissue more organized.</p> <p>Frequent, plump fibroblasts.</p> <p>Disorganized collagen fibers beginning to align across defect.</p> <p>Occasional vascular channels.</p> <p>Osteoclasts adjacent to host bone.</p>	<p>Organized granulation tissue infiltrating defect and surrounding ICBM.</p> <p>Mild, chronic inflammation.</p> <p>Osteoclasts adjacent to host bone.</p>	<p>Granulation tissue surrounding ICBM.</p> <p>Vessels in granulation tissue stroma more organized.</p> <p>Cartilage on dural surface, at and above edges of defect.</p> <p>Decreased mixed inflammatory response.</p> <p>Osteoblasts and osteoclasts adjacent to host bone.</p> <p><b>First evidence of new bone formation.</b></p>
7	<p>Many vascular channels, predominantly capillaries.</p> <p>Osteoclasts associated with edges of host bone.</p> <p>Fibroblasts aligning across defect.</p> <p>More collagen.</p>	<p>Mature granulation tissue in and around ICBM.</p> <p>Frequent vessels.</p> <p>Focally, prominent rounding of stromal cells.</p> <p>Remodeling of calvarial edges more pronounced.</p>	<p>More cartilage, especially at dural interface.</p> <p>Some calcification.</p> <p>Occasional osteoid.</p> <p>Plumping of stromal cells.</p> <p>Granulation tissue surrounding, interspersed within ICBM.</p> <p>Remodeling of host bone.</p>
9	<p>Hypertrophy and calcification of cartilage matrix.</p> <p>Capillary ingrowth.</p> <p>Chondrolysis.</p> <p>Macrophages, multinucleated chondroclasts.</p>	<p>Mature granulation tissue around ICBM; little interstitial activity away from host bone.</p> <p>Cartilage adjacent to host bone.</p> <p>Focal osteoid and cartilage near dural surface.</p> <p>Remodeling of host bone.</p> <p>Giant cells around ICBM.</p>	<p>Osteoid in maturing stroma.</p> <p>Active production of osteoid and cartilage.</p> <p>Cartilage most prominent adjacent to dura and at edges of host bone.</p> <p>Focal osteoid surrounding ICBM.</p>

DAY HETEROTOPIC OG/ICBM	UNTREATED CRANIOTOMY	CRANIOTOMY TREATED WITH ICBM	CRANIOTOMY TREATED WITH OG/ICBM
11 Many osteoblasts, osteoprogenitor cells. New bone formation.	Focal, small islands of bone in connective tissue. Osteoblasts and osteoclasts at bone margins. More mature layer of connective tissue between an immature layer of fibrous connective tissue and dura.	New bone and cartilage in stroma of loose fibrous connective tissue focally becoming dense connective tissue. Mature vessels in stroma. Rounding of stromal cells.	Dense collagen at bone margin. Small fragments of osteoid. Fewer vessels. Hematopoietic tissue at edges of defect. Osteoid and cartilage in more mature stroma. Osteoblasts rimming ICBM.
14 Multinucleated osteoclasts.	Maturing collagen stroma and remodeling of calvarial margins. Focally, small islands of bone within defect.	ICBM in stroma of mature loose fibrous connective tissue which grades to dense fibrous connective tissue. Chondroid and osteoid. Small islands of more mature bone; little surrounding osteoblastic activity. Remodeling of the host margin. Organized collagen.	Exuberant osteoblastic activity from calvarial edges; bridge across defect almost complete. Hematopoietic elements in new bone. Much osteoblast activity, bone around ICBM

DAY	HETEROTOPIC OG/ICBM	UNTREATED CRANIOTOMY	CRANIOTOMY TREATED WITH ICBM	CRANIOTOMY TREATED WITH OG/ICBM
21	Ossicle with hematopoietic bone marrow.	Mature, polarizable collagen across surgical defect. Focal, small islands of bone. Hematopoietic elements at edges of defect.	ICBM in mature fibrous connective tissue stroma. Little to no osteoblastic or osteoclastic activity around ICBM; mild activity at calvarial edges. Small islands of new bone.	Osteoblast activity is prominent. Complete bony bridge across defect. Hematopoiesis; fat.
28		Maturation of stroma. Narrowing of fibroblasts in bridge of collagen. Fewer osteoblasts and osteoclasts at host bone margins.	ICBM in loose to dense fibrous connective tissue. Small islands of bone; continued osteoblast activity. Remodeling at defect margins.	Complete bone bridge, hematopoietic activity.
42		Stromal tissues; focal areas of mild, chronic inflammation; little or no activity at the calvarial margins. Numerous vascular channels.	Islands of bone in fibrous connective tissue stroma that varies from loose to dense. Little to no activity at the calvarial ends.	Maturing bone with fatty marrow.
70			ICBM in loose connective tissue stroma Little activity; few small islands of bone.	Loose fibrous connective tissue; small islands of bone. Hematopoietic marrow in the advancing calvarial ends; few cells remaining in ICBM.

Histomorphometry (Figure 2.) revealed a similar pattern of healing. However, much of the radiopaque material seen at 11 and 14 days was calcified cartilage. This cartilage was replaced by bone by day 18.



a = significantly different from matrix-treated ( $p < 0.05$ )  
 c = significantly different from previous time point ( $p < 0.05$ )

**Figure 2. Bone regeneration detected in coronal sections of 8-mm critical-size defects.** Defects were treated with 25 mg insoluble collagenous bone matrix (ICBM)  $\diamond$ , or 150  $\mu$ g osteogenin reconstituted with 25 mg ICBM  $\blacklozenge$ . Error bars represent one standard deviation.

## DISCUSSION

The initial cellular response at the calvarial defects was similar regardless of the treatment. Polymorphonuclear leukocytes (PMNs) infiltrated the wound, followed by macrophages and fibroblasts. In contrast to heterotopic sites, at bony wound sites the host bone contributes to repair. Osteoclasts began remodeling the host bone margin within 5 days. Osteogenin hastened the production and maturation of cartilage, the appearance of osteoblasts, the production of osteoid

and hematopoietic tissue, and the maturation of bone.

The repair which occurred in response to treatment with ICBM alone is probably the result of osteoconduction; the matrix may act as substratum for infiltrating pluripotential cells. ICBM also acts as a soft tissue spacer, preventing soft tissue prolapse into the wound and subsequent inhibition of repair. The host bone may contribute factors, such as osteogenin which induce the phenotypic expression of cartilage and bone-forming cells. Exogenous OG accelerates this process in a dose-dependent manner (Mark et al. 1990). The actual cellular events through which osteogenin stimulates bone regeneration remain to be elucidated. The results of these experiments indicate that the critical event occurs within the first 5-7 days.

#### **REFERENCES**

Luyten FP, Cunningham NS, Ma S, Muthukumaran N, Hammonds RG, Nevins WB, Wood WI, Reddi AH (1989) Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation. *J Biol Chem* 264: 13377-13380.

Mark DE, Hollinger JO, Hastings CJ, Ma S, Chen G, Marden LJ, Reddi AH (1990). Repair of calvarial nonunions by osteogenin, a bone-inductive protein. *J Plast Reconstr Surg* 86: 623-630.

Reddi AH (1985) Regulation of bone differentiation by local and systemic factors. In: Peck, WA (ed). *Bone and Mineral Research/3*. Elsevier Science Publishers B.V.; pp 27-47.

Sampath TK, Muthukumaran N, Reddi AH (1987) Isolation of osteogenin, an extracellular matrix-associated bone-inductive protein, by heparin affinity chromatography. *Proc Natl Acad Sci USA* 84: 7109-7113.

Takagi K, Urist MR (1982) The reaction of the dura to bone morphogenetic protein (BMP) in repair of skull defects. *Ann Surg* 196: 100-109.