Evaluation of Mesenchymal Stem Cells Following Implantation in Alveolar Sockets: A Canine Safety Study

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Purpose: *The overall goal of this project was to evaluate culture-expanded bone-marrow-derived mesenchymal stem cells (MSCs) for alveolar bone repair in terms of safety and potential efficacy.* ***Materials and Methods:*** *MSCs isolated from bone marrow aspirations were culture-expanded and cryopreserved. Thawed cells were incubated with 3.2 × 5-mm hydroxyapatite/tricalcium phosphate (HA/TCP) cylinders in a closed system containing 5×10^7 cells/mL. Cells alone, cell-free constructs, or cell-loaded constructs were rinsed in saline and implanted in extraction sockets in the mandibular second and fourth premolar sites of 14 beagle dogs. Acute reactions were evaluated histologically after 7 or 21 days, and bone formation was examined after 49 days.* ***Results:*** *Neither implanted MSC-related inflammation nor ectopic osteogenesis was observed. At 7 and 21 days, dil-labeled canine MSCs were found in more than 80% of the implant sites. Few canine MSCs were found in neighboring tissue. Mild inflammation present at 7 days diminished by 21 days. After 49 days, measured bone formation was 34%, 25%, and 35% for cell-loaded, cell-free, and untreated sockets, respectively ($P < .05$). At 21 days, bone formation was evident in all sites. Wound dehiscence was a complication associated with cell exclusionary membranes and resulted in local inflammation.* ***Discussion:*** *The extraction model indicates the safety of MSCs implanted adherent to HA/TCP. Local bone repair occurred in the absence of nonspecific differentiation or migration with distant osteogenesis.* ***Conclusions:*** *An alveolar socket model may be an appropriate model for initial clinical investigation of MSC-mediated bone repair.* Int J Oral Maxillofac Implants 2005;20:511–518

Key words: *alveolar socket model, bone formation, mesenchymal stem cells*

A distinct bone marrow cell population called mesenchymal stem cells (MSCs) gives rise to a variety of mesodermal tissues including bone, cartilage, tendon, muscle, fat, and marrow stroma connective tissue.[1–3] Isolation and culture-expansion methods have been developed.[4] The ex vivo culture-expanded MSCs maintain their multipotential phenotype and are capable of differentiating along various lineages. In vitro, human MSCs express and secrete osteogenic cytokines while retaining their diploid phenotype.[4,5] MSCs have been successfully engrafted within critical-sized defects in the long bones of canines and athymic rats to affect bone repair.[6–9] As evidenced by prior investigations, a good deal is known about MSC biology. It is of interest to translate this knowledge into clinical application.

Several important issues remain unresolved and require elucidation. For example, the delivery of multipotential cells is challenged by the concern for differentiation along undefined and undesired pathways. Additionally, implanted cells might escape the

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site of implantation and lead to tissue formation at unintended sites. The fate of implanted MSCs as part of a therapeutic construct requires definition in a clinically relevant model.

Several relevant models of bone regeneration are available; however, the tooth extraction/alveolar defect model offers several advantages for initial clinical evaluation of bone tissue engineering constructs. Histologic evaluation of human extraction socket healing has demonstrated that bone healing in the alveolar crest occurs as early as 21 days after extraction and that complete repair occurs within 15 weeks. The extraction socket is not a critical-size defect; however, intervention by grafting is advocated clinically. Such interventions have included grafting materials such as autogenous bone, demineralized freeze-dried bone,[10–12] xenogenic bovine bone, and human bone morphogenetic proteins (BMPs).[10–18]

A tooth extraction model for the integrated preclinical and clinical development of a bone tissue engineering construct has several advantages: *(1)* the procedure involved is simple, with limited clinical risk to study participants; *(2)* the socket would protect the construct from mechanical risk; *(3)* the nature of tooth extraction socket healing is well documented; and *(4)* clinical need is common, and thus, there is a related potential for possible recruitment to clinical investigations. The main disadvantage of this model is spontaneous bone regeneration, as the socket is not a critical-size defect.

Based on the successful use of MSCs in a hydroxyapatite/tricalcium phosphate (HA/TCP) scaffold to produce new bone tissue in surgically created defects[19–21] and the use of this HA/TCP scaffold in an ectopic model of MSC-based osteogenesis,[22] this preclinical investigation was performed to define the safety and estimate the efficacy of MSC implantation on an HA/TCP scaffold in tooth extraction and alveolar bone repair. The objectives were to determine the potential for cell migration 7 and 21 days following the implantation of a canine MSC suspension (5×10^7 cells/mL) and to determine by histologic assessment the differentiated fate of HA/TCP-adherent cMSCs 49 days following implantation.

MATERIALS AND METHODS

Cell Preparation

Three or 4 weeks prior to implantation surgery, bone marrow was harvested by iliac crest aspiration from 14 dogs. Nine milliliters of marrow were treated with 1 mL of heparin solution, and within 24 hours, MSCs were isolated using standard procedures.[3,7,23] Following isolation and expansion, cells were cryopreserved at the end of the first passage of the plated cells. For surgery, cells were thawed, rinsed twice by centrifugation/resuspension in ice-cold phosphate buffer solution (PBS), and prepared as cell suspensions for grafting in PBS (5×10^7 cells/mL) or loading of HA/TCP scaffolds (2.0×10^7 mL).

Preparation of Implants

Cryopreserved cells were thawed, and resulting cell suspensions were labeled with the fluorescent dye, chloromethylbenzamido 1,1′-dioctadecyl-3,3,3′,3′ tetramethylindocarbocyanine (CM-dil; Molecular Probes, Eugene, OR). The HA/TCP scaffolds (3.2-mm-diameter × 5 mm; Biomatlante, Nantes, France) were loaded in a closed system under negative pressure with a cMSC suspension (2×10^7 cells/mL). The cMSC-loaded implants were shipped to the animal facility in 1-mL syringes in E38 temperature-controlled boxes (MVE Bio-Medical Division, Chart Industries, Burnsville, MN) at room temperature. The scaffolds were transferred from the 1-mL syringe to a sterile 50-mL conical tube containing 5 mL of DMEM-LG-PF (Dulbecco's Modified Eagle Medium—low glucose—phenol-free; Life Technologies, Gaithersburg, MD). Immediately prior to use, each scaffold was rinsed in 10 mL of saline for 5 minutes.

Animal Care and Surgery

Fourteen adult beagle dogs (Covance, Princeton, NJ) were housed and treated at the University of North Carolina Vivarium following a protocol and procedures approved by Institutional Animal Care and Use Committee. The oral cavity was swabbed with 0.2% chlorhexidine gluconate (Alpharma USPD, Fort Lee, NJ, and Oslo, Norway) prior to surgery. All animals were anesthetized and 2 mandibular premolar teeth (P2, P4) were surgically extracted bilaterally. Mucoperiosteal flaps were closed with sutures, and extraction sites were allowed to heal. Six weeks later, the P2 and P4 sites were exposed using a mucoperiosteal flap, and a 3.5-mm osteotomy was prepared in each root site.

In 4 animals, the sites were covered with a cell-exclusion membrane (expanded polytetrafluoroethylene [e-PTFE], Gore-Tex; W. L. Gore & Associates, Flagstaff, AZ), sutured closed, and filled with 250 to 300 µL of cell suspension (5×10^7 cells/mL. In the remaining animals, 8 sockets were left untreated; each of the remaining 32 osteotomies received one 3.2 × 5-mm construct (with or without MSCs). A cell-exclusion membrane was placed over the implanted site, and the mucoperiosteal site was closed using vertical mattress sutures.

Following surgery, the animals were weighed twice weekly. For the first 48 hours following surgery,

Table 1 Qualitative Histologic Assessment of Labeled cMSC Distribution Following Engraftment

Dog (evaluation time)	Engraftment method	No. of slides evaluated	No. of slides with no labeled MSCs found	No. of slides with labeled MSCs in connective tissue	No. of slides with labeled MSCs in new bone	No. of slides with labeled MSCs in glandular tissue
1 (7 days)	Adherent	7	1	0	0	0
	Suspension	7	5	2	2	2*
2 (7 days)	Adherent	7	6	0	2	0
	Suspension	7	0	0	0	2*
3 (21 days)	Adherent	7	1	0	7	0
	Suspension	7	1	1	3	1
4 (21 days)	Adherent	7	1	0	6	0
	Suspension	7	3	1	2	0

*Single cells were identified among entire field examined.

Rimadyl (1 mg/kg; Pfizer, New York, NY) was given for possible postoperative pain. Polyflex (15 mg/kg; Fort Dodge, Overland Park, KS) was administered intramuscularly for 5 days following surgery. Biomax (5 mg/lb bid; Delmarva Labs, Midlothian, VA) was administered by mouth until suture removal, and each animal received a daily oral mouth rinse using 10 mL of 0.2% chlorhexidine gluconate.

Tissue Harvesting and Necropsy

From the 4 dogs that received cell suspensions, tissues were harvested from 2 at 7 days after placement of cMSCs from 2 at 21 days postplacement. The dogs received 600 mg/lb phenobarbital sodium intraperitoneally and were sacrificed by exsanguination. Animals underwent gross evaluation and histologic examination of the oral palatal mucosa, buccal mucosa, esophagus, and oropharynx as well as histologic evaluation of tissues adjacent to the alveolar defect, including the cell-exclusion membrane and gums. All tissues were fixed in 10% formalin and archived. The 10 canines that received cMSC-containing and cell-free HA/TCP constructs were similarly sacrificed at 49 days following treatment, and their mandibles were harvested bilaterally by excision from the midline to the ramus.

Histomorphometric Analysis

All tissues were fixed for 72 hours in 10% formalin. The mandibles were decalcified, and regions of interest were dissected and embedded in paraffin. Five-μm frontal plane sections were prepared across the entire region of interest (Fig 1). Hematoxylin-eosin (h&e) –stained sections were then subjected to histomorphologic measurement using Bioquant Nova (Bioquant Nova; Bioquant Image Analysis Corporation, Nashville, TN). Of the 40 sockets treated, 6 sockets were excluded because of early clinical identification of infection related to soft-tissue dehiscence, cell-exclusion membrane exposure, and removal or loss of the constructs. One additional dog was excluded because of a cage-chewing habit that caused recurrent trauma to the sutured surgical sites. Thirty sockets were evaluated. The percentage of bone per unit area found in 5 random fields within each extraction socket was measured for all extraction sockets. The average bone/area percentage was calculated for each treatment group and compared using a 2-tailed Student *t* test.

RESULTS

Evaluation of Cell Confinement and Migration

The necropsy and local tissue histology revealed little evidence of cell migration or displacement following placement of MSCs adherent to the HA/TCP scaffold (Table 1). One animal in the group sacrificed 7 days postimplantation and 1 animal in the 21-day group revealed the presence of labeled MSCs in a minor salivary gland tissue in mucosa adjacent to the mandible (Table 1). The evaluation of regional lymph nodes and other tissues by fluorescent microscopy revealed few extravasated or escaped MSCs (Figs 2a and 2b). Dil-labeled MSCs were evident within the HA/TCP matrices in more than 80% of the tooth sockets.

Bone Area

Histologic evaluation of the sites treated with cell suspensions at 7 and 21 days revealed the early osteogenesis associated with dil-labeled cells. The majority of sections for adherent cells at 21 days (Table 1) revealed cells that were resident in bone forming in the extraction sockets (Figs 2c and 2d). At

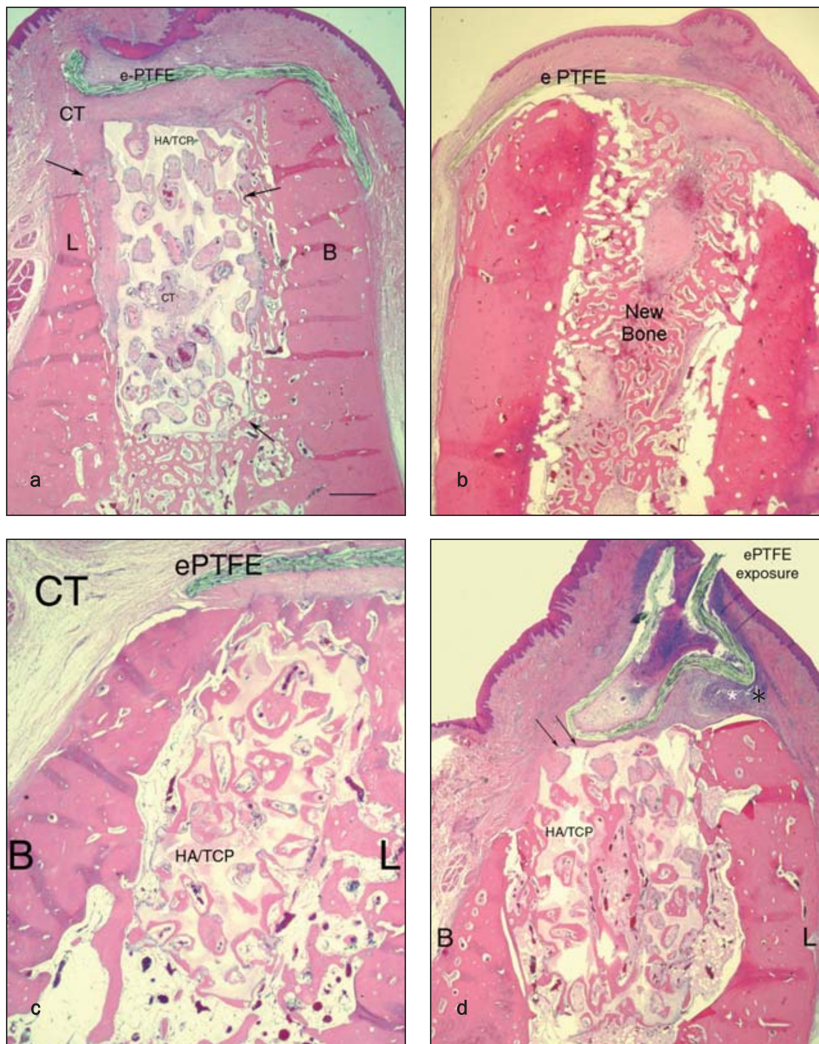

Fig 1 Histologic representation of treated and untreated alveolar sockets 49 days following placement. Frontal sections from the graft site reveal alveolar bone buccal (B) and lingual (L) to the socket and the cell-exclusion membrane (e-PTFE). *(a)* A cMSC-loaded scaffold; the location of HA/TCP scaffold is evident in these decalcified sections. Arrows indicate osteoconduction and new bone formation. Bar = 1 mm. *(b)* Untreated alveolar socket; new bone formation is evident. The e-PTFE cell-exclusion membrane remains intact beneath the mucosa. Bar = 1 mm. *(c)* A cMSC-loaded scaffold; bone formation is noted bridging the socket defect beneath the ePTFE membrane. Bar = 1 mm. *(d)* H&E histologic identification of cell-exclusion membrane exposure (as marked) reveals localized inflammatory infiltrate (*) and scaffold-adherent osteoclasts *(arrows)*, with no other indication of alveolar bone resorption. The MSC-loaded scaffold supported osteogenesis within the pores. (H&E; bar = 1 mm).

49 days, examination of the sites treated with HA/TCP scaffold revealed new bone and osteoid within the scaffold pores of all samples. There was a significant increase in the amount of bone in cMSC-containing scaffolds. For the sites with HA/TCP scaffolds containing cMSC, 34% of the total area of the site was new bone formation area, while sites with cell-free HA/TCP scaffolds displayed 25% new bone formation area ($P < .05$). The untreated sockets contained 35% bone formation in the extraction socket.

Osteogenesis

Osteogenesis occurred in untreated sockets (Fig 1b) as well as within MSC-free and MSC-containing HA/TCP construct-treated sockets (Figs 3a to 3d). Within the central region of the construct, new bone formation was revealed in MSC-containing HA/TCP constructs. Beneath the cell-exclusion membranes and above the MSC-containing constructs, bone formation was frequently observed extending from the buccal to the lingual alveolar host bone (Fig 1c). This did not occur in constructs lacking MSCs. In the residual socket beneath all constructs, woven bone formation was often, but not always, observed.

Osteoconduction

Osteoconduction was observed in all HA/TCP-containing sites, irrespective of the presence or absence of MSCs. Many sections revealed bridging osteogenesis between the socket wall and the HA/TCP scaffold (Fig 1). This process contributed to the bone repair of all filled sockets.

Local Infection and Scaffold Resorption

Local infection associated with placement of the tissue engineering constructs was not revealed where soft

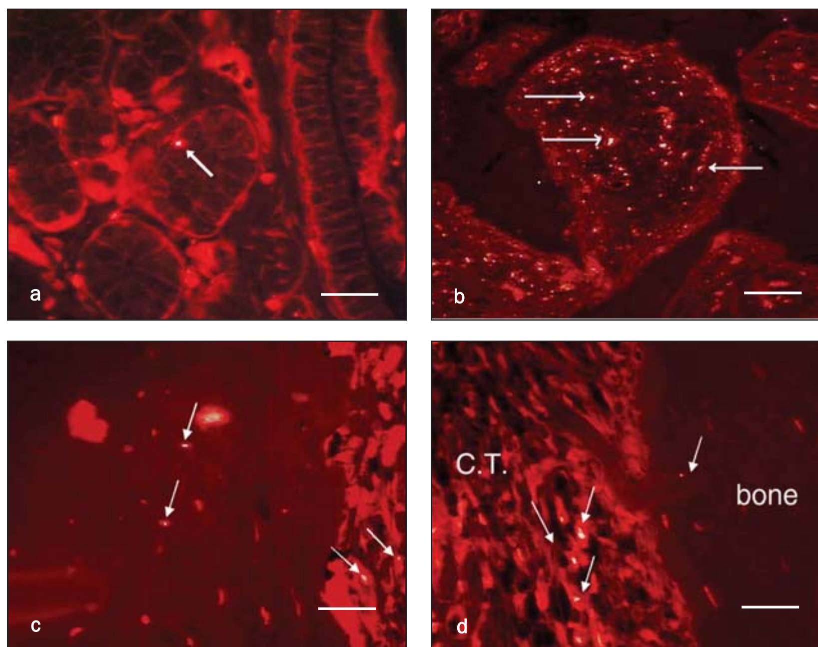

Fig 2 Unstained slides from each implant socket and surrounding soft tissues were analyzed via flourescence microscopy. Immunohistochemical assessment of the engrafted cMSCs after 7 and 21 days. *(a)* A single dil-labeled cMSC was found in salivary gland tissue. The cell was resident between the mucus glands, but had not integrated into the gland nor elicited any adverse response. *(b)* Bone was present in the left P4 extraction socket; dil-labeled cMSCs were prevalent in the new bone filling the HA/TCP scaffold *(arrows)*. *(c)* The right P4 extraction socket contained bone formation and dil-labeled cMSCs embedded within the new bone and forming osteoid. Red color indicates autofluorescence of the collagenous scaffold. *(d)* Bone formation within the HA/TCP implant in the right P2 extraction socket with dil-labeled cMSCs both within the newly formed bone *(arrow)* and in the adjoining connective tissue (CT) (bar = 10 μm).

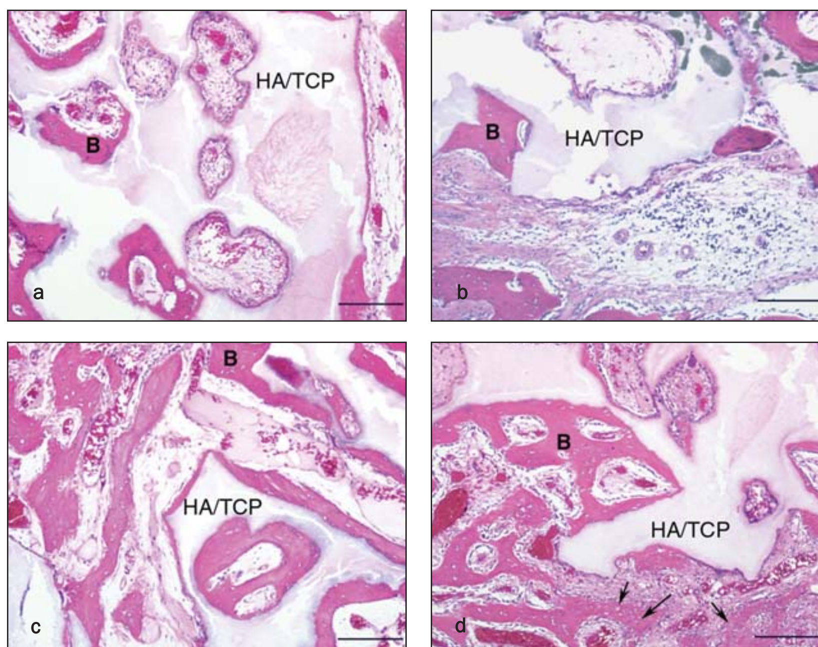

Fig 3 Histologic examination of sockets treated with cMSC-loaded scaffolds *(a,c)* and *(b,d)* cell-free scaffold-treated sockets. Scaffold is indicated *(HA/TCP)*, and new bone formation is marked *(B)*. Osteogenesis is evident within pores and against the scaffold surfaces. Arrows (in *d*) indicate new bone formation arising from the socket wall. (H&E; bar = 200 μm).

tissue closure was clinically evident. Resorption of the HA/TCP scaffold was not observed in the absence of cell-exclusion membrane-related inflammation. In cases where clinical signs of inflammation and exposure of the cell exclusion membrane were not evident, but histologic evidence of soft tissue dehiscence was evident, an acute inflammatory infiltrate was present in the mucosal connective tissue adjacent to the membrane. In these sections, multinucleated cells were observed in contact with the HA/TCP scaffold but not with the adjacent alveolar bone. Bone formation was identified in the pores of such scaffolds and in regions near the adherent multinucleated cells (Fig 4a).

Exposure of the cell exclusion membrane to the oral cavity was associated with clinical signs of local inflammation and histologic evidence of inflammation (Figs 4a and 4b). In this situation, marked resorptive activity was identified by large multinucleated cells adherent to scalloped surfaces of the HA/TCP scaffold.This occurred in 2 samples where clinically

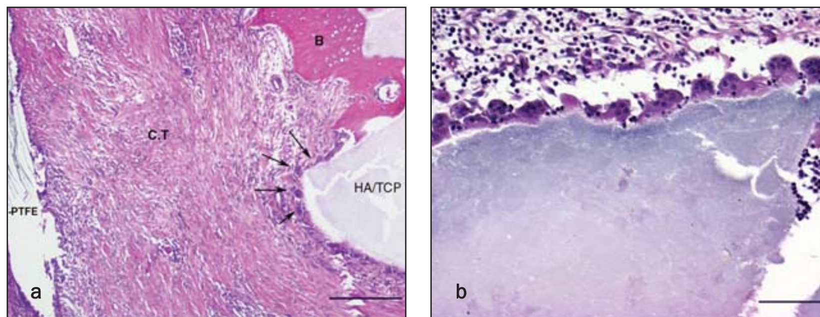

Fig 4 (*a)* Histologic evaluation of microscopically exposed cell-exclusion membranes and treated sockets at 49 days. Low-power examination of the superior aspect of a socket beneath a microscopically exposed ePTFE membrane reveals hyperplastic connective tissue (CT) and a region of the scaffold (HA/TCP) on which bone formation (B) has been achieved and adjacent osteoclast mediated resorption is ongoing *(arrows).* Bar = 200 μm. *(b)* Histologic evaluation of clinically exposed cell-exclusion membranes and treated sockets at 49 days. High-power examination of a cMSC-loaded scaffold located deep within the socket reveals mononuclear cell infiltrate and multinucleated cell (osteoclast) mediate resorption of the HA/TCP scaffold. (H&E; bar = 100 μm).

evident cell-exclusionary membrane exposure was reported throughout the 49-day healing period. Active alveolar bone resorption, indicated by osteoclastic resorption of the surrounding host bone, was not revealed in the associated histologic sections. Interestingly, osteoclasts were observed at the HA/TCP scaffold, but not at the alveolar bone margins.

DISCUSSION

This preclinical model of bone tissue engineering focused on relatively early periods of healing and demonstrated that implanted MSCs were not *(a)* associated with inflammation in neighboring or adjacent tissues, *(b)* displaced by bone formation to neighboring (or distant) ectopic sites, or *(c)* engrafted along other mesengenic pathways. A level of safety was demonstrated.

This study reiterates the ability of MSCs to differentiate along the osteoblastic lineage when implanted adherent to an HA/TCP scaffold. Similar constructs support MSC-directed bone formation in ectopic sites.[22] The contribution of the engrafted cells to this process of bone formation is presently demonstrated in 21-day (Fig 2) sections showing labeled cMSCs in forming bone scaffold. At 49 days, more bone had formed in cMSC-containing constructs than in cMSC-free constructs. This finding is congruent with other observations using human MSCs with similar characteristics.[24] An indirect, osteoinductive effect on host cell bone formation is likely, as MSCs are known to produce at least BMP -2, -4, and -6 during their differentiation along the osteoblastic pathway.[25]

A unique feature of the tooth extraction model is that socket healing is delayed by the placement of osteoconductive biomaterials and osteogenic demineralized freeze-dried bone allograft.[13] This must be considered when reviewing the present results; significantly greater bone area was observed in cMSC-loaded scaffold treated sockets than in the cMSC-free scaffold-treated sockets. Qualitatively, there were no observable differences in the osteoconductive behavior of the MSC-free and MSC-containing scaffolds. Osteogenesis occurred in the central regions and was more frequently observed in the region between the scaffolds and the cell exclusionary membrane for MSC-loaded scaffolds than for MSC-free scaffolds (Fig 1c).

Cartilage or muscle formation following MSC engraftment was absent. Adipogenesis (an event reported during socket healing) was noted, but not in association with the engrafted constructs. Common initial steps along both adipogenic and osteogenic pathways have been suggested from in vitro investigations.[26] Cartilage and fat formation in earlier studies of MSCs adherent to HA/TCP are not commonly reported.[6,22,23] Although MSC differentiation may not utilize a linear pathway of differentiation,[27] MSCs adherent to this HA/TCP scaffold form bone directly.

Inflammation in the absence of healing complications was not observed. Complications encountered were restricted to the habitual cage chewing and recurrent disruption of the suture line of one animal, and mucogingival dehiscence and exposure of the cell exclusionary membranes in 2 animals. Infection was controlled by systemic antibiotics. Systemic infection was not identifiable by malaise or fever in

any animal. These result complications are consistent with reports for cell-exclusionary membrane used in clinical practice.[28,29]

Neither acute abcesses nor chronic granuloma formation were observed. However, wound dehiscence was associated with an inflammatory cell infiltrate and osteoclasts on the HA/TCP scaffold, but not on the host bone. The adherent MSC may play a role in osteoclast recruitment[30] and differentiation and may reflect osteoclast localization to the HA/TCP scaffold. However, engraftment of scaffold-adherent MSCs may not pose substantial risk for localized osteolysis in the event of surgery-related infection.

The initial analysis of cell migration from these constructs or following placement of 5×10^7 cells into sockets alone indicated little potential for migration and subsequent differentiation. Thus, use of a cell exclusionary membrane to prevent migration of MSCs from the site of placement could be eliminated from future investigations.

CONCLUSIONS

Bone marrow stroma-derived multipotential cells or MSCs are capable of expansion and maintained multipotentiality. When placed in orthotopic tooth extraction sites, MSCs adherent to an HA/TCP scaffold remained in the specified site and contributed to bone formation as differentiated osteoblastic cells. cMSC-containing scaffolds demonstrated greater formed bone area than MSC-free scaffolds. Evidence for differentiation along other mesenchyme-derived lineages (eg, chondroblastic, myoblastic) was not revealed by the histologic assessment. Local inflammation resulting from soft tissue dehiscence and cell exclusion membrane exposure precluded bone formation but did not result in marked alveolar bone resorption or unspecified differentiation of the implanted MSCs. This preclinical evaluation suggests that a similar clinical model could provide necessary clinical and histologic safety data and initial efficacy data necessary for bone tissue engineering development.

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