Characterization of ViviGen Formable™ Cellular Bone Matrix

Abstract

Bone repair is a multifaceted process that requires osteoconductive, osteoinductive, and osteogenic elements working in concert to drive new bone formation. ViviGen Formable™ Cellular Bone Matrix is a commercially available cellular allograft designed to provide each of these elements. It contains: 1) Demineralized bone to provide osteoinductive signaling; 2) Corticocancellous chips to provide an osteoconductive scaffold for cell attachment, proliferation and migration; and 3) Viable, lineage committed bone cells to provide osteogenicity. These three key components are necessary for the bone repair process. ViviGen® Cellular Bone Matrix is the first cellular allograft focused on protecting viable, lineage committed bone cells from recovery to implantation. The purpose of the investigation was to assess ViviGen Formable for the osteoconductive, osteoinductive, and osteogenic properties necessary for bone healing. Data from enzyme-linked immunosorbent assays (ELISA) demonstrated that ViviGen Formable demineralized bone fibers contain the appropriate physiological levels of growth factors (BMP-2 and BMP-7) capable of osteoinductive signaling. This osteoinductive (OI) potential was further tested in vivo in an OI athymic nude mouse model. The results showed the demineralized bone fibers drove the formation of new bone elements complete with deposition of bone matrix as well as the formation of both bone marrow and blood vessels. Furthermore, in vitro studies demonstrated that ViviGen Formable demineralized bone fibers provide a biocompatible scaffold that support cell attachment, infiltration and colonization, and also cellular proliferation. Importantly, this study illustrated that the lineage committed bone cells found in ViviGen Formable can attach and thrive on this new demineralized bone fiber. Mesenchymal stem cells (MSCs) also attached and colonized the demineralized bone fibers. This data together suggest that osteoblasts and osteocytes enriched in these areas may provide the cellular signaling that drives MSCs to become lineage committed bone forming cells even further facilitating the bone formation process.
In summary, the demineralized bone fibers that contribute to the moldable handling characteristics of ViviGen Formable have demonstrated the osteoinductivity and osteoconductive properties that promote bone healing.  

KEY WORDS: demineralized bone fibers; osteoinductivity; growth factors; cell attachment; bone growth; ViviGen Formable

Introduction

The ability of demineralized bone to help facilitate bone healing has been known in clinical settings for over a century. However, the factors contributing to this phenomenon were unknown until 1965 when Dr. Marshall Urist characterized specific active proteins trapped in the matrix of bone that endow it with this property. These protein factors are called bone morphogenetic proteins (BMPs) while the property of directing bone formation is referred to as osteoinduction. Since this discovery, physicians performing orthopedic and spinal fusion surgeries have frequently used bone void fillers containing demineralized bone sourced from donated human tissue to promote the healing process and formation of new bone. More recently, researchers have examined the effect of adding cellular components to bone void fillers to additionally provide an osteogenic component. These living cells can help build upon the osteoinductive potential of demineralized bone and further facilitate the bone healing process. There is now a further paradigm shift in the field of bone and tissue repair resulting in the focus on lineage committed bone cells as the preferred cell type. Preclinical evidence has demonstrated in vivo that implanted bone forming cells not only persist at the sites where they were implanted, but also directly contribute to mature bone formation. Other favorable qualities that lineage committed bone cells may demonstrate include: 1) stimulating vascularization through IGF-1 mediated mechanisms, 2) attracting the host’s own bone forming cells to sites of implantation through chemotactic mechanisms, and 3) driving the likelihood that mesenchymal stem cells (MSCs) will differentiate into lineage committed bone cells that may also directly participate in forming new bone. The findings of these studies formed the rationale for the development of ViviGen (LifeNet Health, Virginia Beach, VA). ViviGen is comprised of osteoinductive, demineralized bone particulate and osteoconductive corticocancellous bone chips that house the osteogenic lineage committed bone cells. The second generation, ViviGen Formable, has the same osteogenic and osteoconductive components as ViviGen, but utilizes precision machined, demineralized bone fibers to achieve a more moldable handling
quality. The purpose of this study was to assess whether these demineralized bone fibers possess the appropriate growth factors for osteoinductivity, demonstrate \textit{in vivo} osteoinductivity, and support \textit{in vitro} cell attachment, cellular proliferation, and viability.

**Methodology Overview**

1. **Fiber Generation:**
   Human cortical bones were recovered from six donors with research authorization through LifeNet Health’s organ and tissue procurement service. Femurs were debrided and the marrow and trabecular bone were removed. The resulting bone segments were cut into fibers, processed, and demineralized utilizing proprietary procedures developed by LifeNet Health.\

2. **Fiber Analysis:**
   a. \textit{In vitro} Growth Factor Analysis
   Demineralized bone fiber lots (derived from 6 donors) were analyzed for the presence of BMP-2 and BMP-7 content \textit{in vitro} by first digesting the tissue with a collagenase solution at 37°C to facilitate the extraction of proteins. The resulting solutions were analyzed in triplicate for the presence of BMP-2 and BMP-7 via ELISA (R&D Systems, Indianapolis, Indiana). Results are reported as ng protein/g of demineralized bone fibers.

   b. \textit{In vivo} Osteoinductive Potential (OI)
   Osteoinductive potential (OI) and the generation of new bone were assessed \textit{in vivo} utilizing a 35 day athymic mouse muscle pouch model of OI. Four replicates (20-25g) of freeze dried demineralized bone fibers were weighed out from lots separate from those used in the growth factor analysis and cell attachment studies, rehydrated in saline, and loaded into a syringe for delivery. The rehydrated sample was used within 5 minutes of preparation and implanted inter-muscularly between the biceps femoris and gluteus superficialis muscles of athymic mice. The implants were recovered at 35 days post-implantation and processed for histological microscopic assessment of hematoxylin and eosin (H&E) stained sections. The osteoinductivity of the implanted bone fibers was determined by the presence of bone elements and bone matrix deposition.

   c. \textit{In vitro} Cell Attachment and Sustained Cellular Proliferation
Demineralized bone fibers (6 donor lots previously analyzed for growth factors) were placed in cell culture and seeded with either previously frozen ViviGen derived bone cells, or thawed bone marrow-derived mesenchymal stem cells (BM-MSCs) recovered from human donor bone marrow. Prior to experimentation, the BM-MSCs were identified on the basis of cell surface marker expression via flow cytometry. The osteoblasts and BM-MSCs were seeded separately at 62,500 cells per 60.5 ± 1 mg fiber sample on day 0 and cultured over the course of 7 days in their respective growth media. These cultures were examined at 1 hour, 1 day, and 7 days post-seeding by scanning electron microscopy (SEM) to assess cell attachment and concomitantly by Alamar Blue staining to assess sustained cellular proliferation.4

Results

Presence of Growth Factors and Osteoinductivity

Using a proprietary demineralization process, bone fibers generated from 6 individual donors were processed and freeze dried. The resulting demineralized fibers were subsequently treated with a collagenase solution to digest the tissue and facilitate the extraction of proteins for analysis. Specifically, the BMP-2 and BMP-7 (Figure 1) content were measured in triplicate utilizing ELISA. Figure 1 depicts the average BMP-2 and BMP-7 levels from six donor samples.2 Other studies have reported a wide span of BMP-2 and BMP-7 levels in demineralized bone, with ranges from 6.5-110 ng and 44-125 ng per g demineralized bone, respectively.15-17 The data presented here demonstrates that BMP-2 and BMP-7 were present, and fell within expected levels, in the demineralized bone fibers of ViviGen Formable after processing.6
**Figure 1.** Average level of BMP-2 and BMP-7 content in ViviGen Formable demineralized bone fibers from six donor samples, each performed in triplicate, as measured by ELISA.

After demonstrating *in vitro* that new ViviGen Formable demineralized bone fibers contain BMP-2 and BMP-7, demineralized bone fibers generated in the same manner were intra-muscularly implanted into athymic nude mice to test their *in vivo* osteoinductivity (OI). After 35 days, the implanted material and surrounding tissue were removed and histologically prepared for hematoxylin and eosin (H&E) staining. Images from an H&E stained sample are presented in **Figure 2 (A-C).** **Panel A** shows a set of merged images that robustly illustrate more than 50% new bone elements present in the entire ectopic site (4x magnification). **Panels B** and **C** highlight at 4 and 10x magnification, respectively, the presence of new bone elements such as bone marrow (**&**), new vascularization (**^**), osteoblasts (**□**), chondrocytes (**%**), and new bone (*****) dispersed amongst the remaining implanted demineralized fibers (**#**). These observations demonstrate the new ViviGen Formable demineralized bone fibers retain OI potential: They not only support the colonization of cells, but also the formation of new bone and bone marrow, as well as neo-vascularization, an important aspect of the healing process.³
Figure 2. ViviGen Formable demineralized bone fibers demonstrated osteoinductivity in athymic nude mice. Panel A shows a set of merged H&E images that demonstrate more than 50% new bone elements present in the entire ectopic site at 35 days post-implantation (4x magnification). Panels B and C are also H&E images shown at 4 and 10x magnification, respectively, that depict the presence of new bone elements such as bone marrow (&), new vascularization (^), osteoblasts (□), chondrocytes (%), and new bone (*) dispersed amongst the remaining implanted demineralized fibers (#) at 35 days.

Support of Cellular Attachment

The next set of experiments were designed to examine in vitro the colonization of the demineralized bone fibers by human bone marrow-derived mesenchymal stem cells (MSCs) and also bone cells derived from human corticocancellous bone. The purpose of these experiments was to model bone cell migration from the corticocancellous chips to the demineralized bone fibers. Additionally, this may also represent how a
patient’s own bone forming cells might interact with the demineralized bone fibers in ViviGen Formable. Demineralized bone fibers generated from the original 6 donor lots were stored in a cryopreservative. The fibers were treated in this manner to model the ViviGen Formable product. Prior to their use, the demineralized bone fibers were thawed and rinsed per the same instructions for preparing ViviGen Formable for implantation. Previously isolated and frozen ViviGen derived bone cells were thawed, cultured, and seeded along with the demineralized bone fibers at a density of 62,500 cells/60.5 ± 1 mg demineralized bone fibers in low-attachment cell culture wells.\(^4\)

Another cell population that supports new bone formation are mesenchymal stem cells (MSCs). To model how a patient’s own MSCs might interact with the new demineralized bone fibers, human bone marrow-derived mesenchymal stem cells (BM-MSCs) were also separately cultured with ViviGen Formable demineralized bone fibers.\(^4\)

After 1 hour, 1 day, and 7 days, the samples were prepared for SEM analysis. Representative images at 3000x magnification are shown in Figure 3. Cells are highlighted in the figure by the asterisks (*). As seen in the images, both ViviGen derived bone cells and BM-MSCs attached to the ViviGen Formable demineralized bone fibers. As expected, both cell populations spread and formed processes over 7 days. The colonization of the fiber scaffolds by networks of cells confirm that they provide a biocompatible environment in supporting two cell types (i.e., ViviGen derived bone cells and BM-MSCs) that are involved in the process of generating new bone.\(^4\)

![Figure 3](#)
**Figure 3.** ViviGen Formable demineralized bone fibers support cellular attachment of both ViviGen derived bone cells and human BM-MSCs. Over the course of 7 days, cells (*) were observed spreading along the fibers, elongating processes, and interacting with other networks of cells.

**Support of Sustained Cellular Proliferation and Viability**

To further confirm that the new demineralized bone fibers in ViviGen Formable provide a biocompatible environment for bone cells, an Alamar Blue assay was used to measure cellular proliferation of ViviGen derived bone cells seeded on demineralized bone fibers. The data illustrates that following seeding on day 0, the ViviGen derived bone cells demonstrated increasingly robust levels of cellular proliferation when cultured with ViviGen Formable demineralized bone fibers over the course of 7 days.\(^4\)

**Figure 4.** The results of an Alamar Blue assay showed that ViviGen Formable demineralized bone fibers supported cellular proliferation with ViviGen derived bone cells. The results show averages from 6 donor samples (A-F) conducted in triplicate. RFU = relative fluorescent units.

**Summary**

ViviGen Formable demineralized bone fibers were analyzed for multiple properties. The profile of growth factors (BMP-2 and BMP-7) present in these fibers were within the expected physiological ranges similar to levels reported in the literature for other demineralized bone matrix. Their presence suggests appropriate osteoinductive signaling potential necessary for new bone formation. Further *in vivo*
confirmation of osteoinductivity was observed in an athymic mouse model in which the fibers functionally supported the robust formation of new bone elements complete with deposition of bone matrix including both bone marrow and blood vessels.

ViviGen derived bone cells were used as the osteogenic component to further test the fibers. The data demonstrated that these human cells both attached to and colonized the fibers and their cellular proliferation and viability could be sustained over time. Not only did these cells demonstrate matrix deposition potential, but the literature also shows their ability to produce chemotactic factors that attract additional osteoblasts, such as the patient’s own osteoblasts, to sites where they are implanted and which may further contribute to new bone formation.\textsuperscript{14}

Notably, human -MSCs seeded on the demineralized bone fibers also successfully attached to and colonized the demineralized bone fibers as shown by SEM analysis. MSCs that migrate and colonize the demineralized bone fibers \textit{in vivo} may be driven to differentiate to a committed bone lineage by factors secreted by both osteoblasts and osteocytes.\textsuperscript{5} However, the process that an MSC undertakes to become a bone cell may take a significant amount of time (21 days or more \textit{in vitro}), and therefore it is likely that the lineage committed bone cells are initially the main contributors to the bone formation process, while the MSCs may contribute more indirectly or secondarily over time.\textsuperscript{5,18} The data showed that MSCs could colonize the demineralized bone fibers \textit{in vitro} suggesting the likelihood that MSCs could remain in the areas enriched with the signaling of osteoblasts and osteocytes long enough to drive MSC differentiation into bone forming cells. In addition, this process could be further supplemented with the osteoinductive signaling of the demineralized bone fibers.

\textbf{Conclusion}

ViviGen Formable demineralized bone fibers were shown to be a biocompatible scaffold that provides the infrastructure and signaling to support the culture of both bone cells and MSCs. These cells were shown to adhere, form networks, and functionally deposit extracellular matrix on the scaffold. This creates an environment whereby ViviGen derived bone cells may participate in bone healing as well as attracting the patient’s own population of osteoblasts to the site of implantation.

The results presented here demonstrate that the demineralized bone fibers in ViviGen Formable exhibit the osteoinductive potential and osteoconductive properties needed to promote bone formation while also contributing moldable handling properties to this cellular bone allograft.
References:

1. DHF# 15-001
6. Data on file LifeNet Health: DHF 15-001