PREMIERE ISSUE

www.bioprocessingjournal.com

BioProcessing

Technical Developments and Opportunities

A publication of
The Williamsburg BioProcessing Foundation

Vol. 1 No. 1

March 2002

Local Gene Therapy for Lumbar Spine Fusion

BY JEFFREY C. MARX

Portions of this article were presented at the Williamsburg BioProcessing Foundation, Cell & Tissue BioProcessing Conference, Williamsburg, VA, Nov. 2001.

In orthopedic procedures, there is a need to form new bone to repair and fill defects arising from either trauma or degenerative disease. The current standard treatment utilizes an autograft,usually from the iliac crest, which results in a second surgical site, weakness in the harvest or donor harvest area, and additional patient morbidity. The goal of our research is to employ a local, ex vivo, gene therapy to obviate the need for autograft in spine fusion procedures.

The LIM Mineralization Protein (LMP-1) is a novel intracellular protein capable of inducing bone formation in vitro and in vivo. In this article, I will outline a rapid protocol, whereby buffy coat cells, isolated from autologous peripheral blood, are transduced with an adenoviral vector encoding the LMP-1 gene. These transduced cells are then implanted on a collagen carrier to promote posterolateral arthrodesis.

Introduction

Spinal fusion is defined as the elimination of movement across an intervertebral motion segment, and is performed to limit motion and reduce pain in the spine by fusing two or more vertebrae. Fusion is performed in cases of injury to spinal vertebrae, protrusion and degeneration of intervertebral disk, curvatures (such as scoliosis or kyphosis), and weak or unstable spines caused by infections or tumors.

Fusion currently is accomplished surgically through the application of bone graft and/or spinal instrumentation. There are approximately 500,000 bone graft procedures performed annually in the United States, 50% of which are spinal fusions. However, the percentage of cases that fail to produce a solid bone fusion (pseudoarthrosis) ranges from 5 to 35%.[1,2] The current standard fusion treatment utilizes an autograft (usually harvested from the iliac crest), that results in a second surgical site, weakness in the donor harvest area, and up to an additional 30% patient morbidity.[3]

In order to obviate the need for a second surgical site, with its incumbent side effects, and to decrease the incidence of pseudoarthrosis, we are exploring a local gene therapy approach to induce bone growth for spinal arthrodesis. To achieve this we need to initiate a series, or cascade of osteoinductive proteins, including BMPs, at physiological dosages that can result in local induction of controlled bone formation.

This brings us to the protein and gene of choice, LMP-1. The LIM Mineralization Protein-1 (LMP-1) is a novel intracellular LIM domain protein capable of inducing bone formation in vitro and in vivo.[4-6]

LMP-1 was first identified in messenger RNA obtained from rat calvarial osteoblasts that had been stimulated with glucocorticoid by Dr. Scott Boden's group at Emory University.[7] Its sequence was determined using an osteosarcoma complimentary DNA (cDNA) library. In situ hybridization results map LMP-1 expression during embryological bone formation, temporally and spatially, with bone morphogenic protein 6 (BMP-6), indicating involvement in the BMP signal cascade. These findings, coupled with in vitro antisense oligonucleotide inhibition and overexpression studies, clearly indicate that LMP-1 is an essential intracellular positive regulator for osteoblast differentiation.[7]

Unlike BMPs, which are secreted proteins acting through cell surface receptors, LMP-1 appears to be an intracellular molecular signal that must be synthesized in situ to induce an osteogenic effect.[8] Therefore, any in vivo use of LMP-1 to induce osteogenesis would need to be a gene therapy approach, delivering the LMP-1 gene into the target cells where expression of the LMP-1 protein can initiate the bone formation process.

This gene therapy approach for spinal fusion is less problematic than other gene therapy approaches since it requires localized gene expression for a limited time (less than seven days).

Therefore, the strategy is to perform an ex vivo transduction of autogenous target cells, using a non-integrating transient expression vector containing the LMP-1 cDNA, and then reintroduce the cells into the recipient in a manner that limits their systemic distribution. If successful, this strategy may prove to be a feasible alternative for bone induction utilizing implantation of pharmacological doses of recombinant osteoinductive proteins.

Methods

Vector Construction

The experiments utilize a replication deficient (DE1/DE3) recombinant human serotype 5 adenovirus, with either the rat (AdV5-rLMP-1) or human (AdV5-hLMP-1) homologues of LMP-1

***Jeffrey C. Marx, Ph.D.** is manager, biological research at Medtronic; Sofamor Danek, Memphis, TN 38132, jmarx@sofamordanek.com.*

cDNA driven by a CMV promoter. These vectors were produced according to the manufacturer's protocols supplied in the Adeno-Quest Kit (Quantum Biotechnologies, Quebec, Canada).

Recombinant plaques were identified by polymerase chain reaction (PCR) for LMP-1 cDNA, and by functional assay. The selected virus was propagated in 293A cells purified using double cesium chloride gradients, and titered by plaque assay.[9]

Buffy Coat Isolation and Transduction

Peripheral blood samples, composed of 3ml of blood plus 0.5ml heparin saline solution, was added to a 50ml conical tube containing 17ml of αMEM media (Gibco BRL, Gaithersburg MD) and 20U/ml heparin. Samples were either autologous for rabbit fusions, or xenogeneic for the athymic ectopic rat implants. All samples were separated at room temperature in a centrifuge at 1100 x g for 10 minutes. The plasma layer was aspirated and the buffy coat layer was collected. A manual cell count was performed and the buffy coat cells were divided into suitable aliquots for transduction.

At varying virus to cell ratios, viral transduction was performed at 37°C for 10 minutes. Samples for rabbit spinal fusions were transduced in 1.0ml volumes while rat ectopic samples were transduced in 0.1ml volumes.

Animal and Surgical Protocols

All in vivo protocols were approved by the institutional animal care and use committee.

Rabbit Spinal Posterolateral Arthrodesis and Analysis

Adult female New Zealand white rabbits, weighing between 4 and 4.5 kg and approximately one year of age, were used in all spinal procedures. The rabbits were housed one per cage, allowed ad libitum diet of rabbit chow and water, and inspected daily for general health and neurological condition. Using a posterolateral intertransverse process, a single level lumbar arthrodesis was performed at L5-L6, using the bilateral paraspinal muscle splitting approach.[10]

After decortication of the transverse processes using an electric bur (Stryker Instru ments, Kalamazoo), 4 million transduced cells, suspended in 2.0 ml of αMEM media, were loaded onto carrier material and placed into the transverse process bed. The rabbits were sacrificed after four weeks; and their spines were excised and evaluated by manual palpation, radiographs, computed tomography scans, and non-decalcified histology.

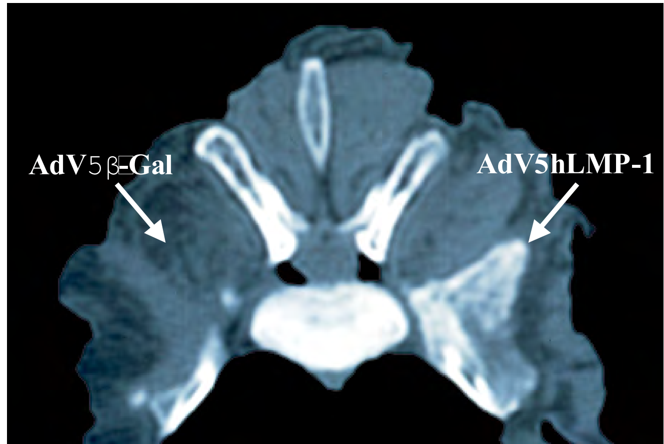

Figure 1. Computed tomographic scans of the lumbar spine from rabbits- 4 weeks after arthrodesis with 4 million buffy coat cells transduced with AdV5hLMP-1 (right) AdV5βGal (left) at an MOI = 4 for 10 minutes. This section is through the intervertebral space. The fusion mass generated by the cells transduced with AdV5hLMP-1 is clearly visible on the right. Noted is the absence of any bone formation on the left side that was arthrodesed with the same carrier loaded with cells transduced with AdV5βGal.

Modeling and Overcoming an Adenovirus Immune Response

Adult NZW rabbits (N=18) were injected intraveniously with 10^8 viral particles of AdV5-βGal. The rabbits were housed one per cage, allowed ad libitum diet of rabbit chow and water, and inspected daily for general health and neurological condition.

Four or sixteen weeks post immunization, a single level lumbar arthrodesis (posterolateral intertransverse process) was performed at L5-L6, using the previously detailed, bilateral paraspinal muscle splitting approach.

At four weeks (N=6) and sixteen weeks (N=3) post immunization, four million autologous buffy coat cells were transduced, ex vivo for 10 minutes, with AdV5hLMP-1 at an MOI of 4.0, Then the transduced cells were implanted on collagen carriers to promote posterolateral lumbar arthrodesis. Non-immunized rabbits (N=9) underwent identical surgical procedures. Additional immunized rabbits (N=3) underwent arthrodesis at 4 weeks with increased cell number ($10x10^6$), an increased viral dose MOI=10 (N=3), or with both parameters increased (N=3). All animals were euthanized at 4 weeks post-arthrodesis, and their spines were assessed by palpation, radiography, and non-decalcified histology.

Characterizing LMP-1 Induced Osteogenesis using Ectopic Implants

Athymic rats are anaesthetized by intraperitoneal injection of ketamine hydrochloride (90mg/kg) and xylazene (10mg/kg). After a 10mm incision was made on the chest wall and a pocket was developed by blunt dissection, each subcutaneous collagen implant was inserted. Four pockets were generated on each rat—two on either side of the sternum. Implants were loaded with one million rabbit, or human, buffy coat cells that had been transduced for ten minutes (MOI = 4) with either AdV5hLMP-1 or AdV5βGal, and then placed in the dissected pouches. The skin was closed with resorbable suture.

Animals were euthanized and the implants were removed on days 1, 3, 5, 7, 10, 14, 21 and 28 post-surgery. The implants were assessed by non-decalcified histology.

Palpation

Manual palpation of the spine was performed at the time of excision, and served as a preliminary evaluation of the fusion status. Palpation consisted of probing the fusion mass for size and density, as well as evaluating rotation about the fused intervertebral motion segment.

Table 1. Arthrodesis study		
Virus	MOI	Fusion
AdV5hLMP1	4	10/10
AdV5bGAL	4	0/10

Table 1. Pivotal rabbit arthrodesis study performed on twenty NZW rabbits. Ten rabbits were arthrodesed with collagen implants loaded with 4 million buffy coat cells transduced with AdV5hLMP-1 at an MOI=4 for 10 minutes. The other four were arthrodesed with implants loaded with 4 million buffy coat cells transduced with AdV5bGal at an MOI=4 for 10 minutes. All ten animals arthrodesed with the AdV5hLMP-1 transduced cells had solid bilateral fusions as evidenced by manual palpation, radiography and histology. None of the ten animals treated with the AdV5bGal transduced cells evidenced any fusion or bone formation.

Table 2A. Successful fusions/attempted fusions			
Implant Composition	Naive Rabbits	4 Weeks Post-Immun.	16 Weeks Post-Immun.
4M Cells MOI = 4	9/9	0/6	2/3

Table 2a. Bone formation in naïve immune competent and immunized rabbits. Immunized animals received an IV injection of 10^8 viral particles of AdV5bGal. Four weeks post immunization nine naïve and six immunized animals were arthrodesed with collagen implants loaded with 4 million autologous buffy coat cells transduced with AdV5hLMP-1 at an MOI=4 for 10 minutes. Sixteen weeks post immunization an additional three animals were arthrodesed with collagen implants loaded with 4 million buffy coat cells transduced with AdV5hLMP-1 at an MOI=4 for 10 minutes. All animals were euthanized four weeks post arthrodesis and their spines assessed by manual palpation, radiography and histology.

Table 2B. Successful fusions/ attempted fusions		
Cells	MOI=4	MOI=10
4M	0/6	0/3
10M	2/3	3/3

Table 2b. Bone formation in AdVbGal immunized rabbits. Immunized animals received an IV injection of 10^8 viral particles of AdV5bGal. Four weeks post immunization, immunized animals were arthrodesed with collagen implants loaded with 4 million autologous buffy coat cells transduced with AdV5hLMP-1 at an MOI= 10 (N=3), 10 million autologous buffy coat cells transduced with AdV5hLMP-1 at an MOI = 4 (N=3), or 10 million autologous buffy coat cells transduced with AdV5hLMP-1 at an MOI= 10 (N=3). The six animals in the 4 million cells and MOI=4 group are those listed in Table 2a. All animals were euthanized four weeks post arthrodesis and their spines assessed by manual palpation, radiography, and histology.

Radiographic Analysis

Rabbit spines were evaluated with posteroanterior radiographs. The post-mortem radiographs were reviewed in a blinded manner, and were graded by two observers as fused or not fused, based upon the presence of continuous bone bridging between, and incorporating onto, the transverse processes.

Computed tomographic scans of the lumbar spine were performed on a high speed CT Scanner (General Electric, Milwaukee, WI) with parameters of: 10cm field of view, 150mAs, 110kV, 1mm gap, and 1mm slice thickness.

Non-Decalcified Histology

Excised lumbar spines and subcutaneous ectopic implants were fixed for 24 hours in 10% neutral formalin, and then transferred to 70% ethanol. After fixation, the specimens were trimmed, dehydrated in 95% and 100% ethanol, and cleared in xylene. Specimens were embedded in methylacrylate and sectioned axially to 5µm thickness on a microtome (Jung Model E Polycut, Leica, Deerfield, IL).

For basic cell morphology, the sections were stained either with Goldner Trichrome to highlight the mineralized bone (blue) or unmineralized tissue (red), or with haematoxylin and eosin (H & E).

Results

Rabbit Spinal Posterolateral Arthrodesis and Analysis

Preliminary studies were performed to optimize the transduction parameters, including viral dose and transduction time, as well as the number of target cells per implant. These values were established at $4x10^6$ cells per implant that were transduced at an MOI = 4 for 10 minutes.

In these preliminary studies, animals were implanted on one side of the spine with cells transduced with the AdV5hLMP-1 virus, while the other side received cells transduced with AdV5βGal.

At four weeks post surgery, the animals were sacrificed. The excised spines showed unilateral fusion masses that were deemed solid upon manual palpation of the spine motion segment, and had plain radiographs that demonstrated bridging bone throughout the fusion mass, including incorporation into the transverse processes. Computed tomography scans (CTs) through the fusion masses revealed solid mature cancellous bone on the side receiving the AdV5LMP-1, but no mass was detected on the side receiving cells transduced with the AdV5βGal virus (see Figure 1).

Non-decalcified histology revealed normal trabecular bone formation in the fusion masses. Sites receiving cells that were not transduced with the active LMP-1 cDNA showed near complete resorption of the collagen carrier.

The pivotal, in vivo, rabbit spine arthrodesis was conducted on twenty NZW rabbits. All protocols were the same in this study, with the exception of a change to a collagen carrier containing ceramic granules.

Ten animals received AdV5hLMP-1 transduced cells, and the remaining ten received AdV5βGal transduced cells as a negative control. All twenty animals survived until completion of the study. One rabbit exhibited a sub-clinical, unilateral infection, but still formed a continuous fusion mass.

All 10 animals that received the AdV5hLMP-1 transduced buffy coat cells had bilateral fusion masses that were deemed solid upon manual palpation of the spine motion segment. Plain radiographs revealed evidence of new bone formation in the AdV5hLMP-1 treated animals. However, due to the

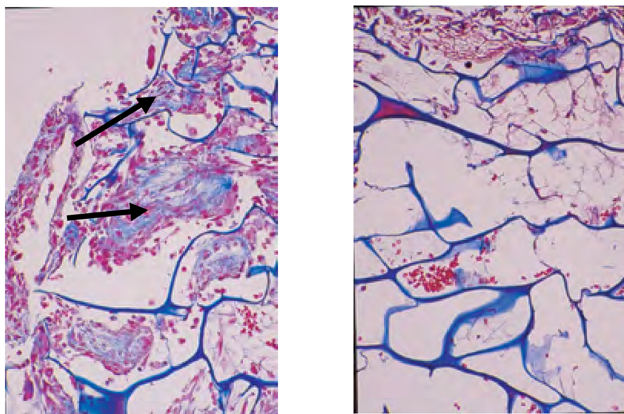

Figure 2. Goldner Trichrome stained sections of ectopic implants excised from athymic rats at 5 days post implantation. Newly formed bone stains blue while non-mineralized tissues stain red. Implants were loaded with 1 million cells transduced with either AdV5hLMP-1 (left) or AdV5βGal (right) at an MOI = 4 for 10 minutes. In the left panel (AdV5hLMP-1) arrows indicate areas of initial bone formation (blue). The right panel showed a lack of bone formation and a reduced cell accumulation in the implant loaded with AdV5βGal transduced cells.

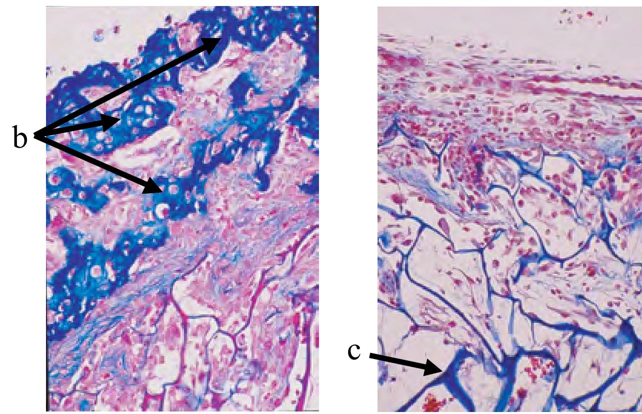

Figure 3. Goldner Trichrome stained sections of ectopic implants excised from athymic rats at 7 days post implantation. Implants were loaded with 1 million cells transduced with either AdV5hLMP-1 (left) or AdV5βGal (right) at an MOI = 4 for 10 minutes. In the left panel (AdV5hLMP-1) there is an obvious formation of bony trabeculae (b). The right panel showed a lack of bone formation and a reduced cell accumulation in the implant loaded with AdV5βGal transduced cells. The blue strands (c) are the collagen fibers comprising the carrier.

radiopaque nature of the ceramic still present after four weeks, it was difficult to make a reliable assessment of the fusion mass, based solely on these films.

CTs were obtained on all spines that had plain radiographs and demonstrated bridging bone throughout the fusion mass, including incorporation into the transverse processes. None of the animals receiving cells transduced with the AdV5βGal formed spine fusions (see Table 1).

Modeling and Overcoming an Adenovirus Immune Response

All non pre-immunized animals (N=9) that received four million cells per implant, transduced at MOI =4, had solid spine fusion masses. These were deemed solid upon manual palpation of the spine motion segment, and had plain radiographs that demonstrated bridging bone throughout the fusion mass, including incorporation into the transverse processes.

None of the six animals achieved fusions that were arthrodesed at four weeks following immunization, and had received four million cells per implant transduced at MOI =4. While at sixteen weeks post immunization, two of the three rabbits that had received the same number of transduced cells achieved fusions. Four weeks post immunization, an increase in the viral dose (MOI = 10) did not overcome the immune response (0/3 fused). However, increasing the cell number to ten million resulted in successful fusion in two of the three animals (2/3), while increasing both viral dose and cell number resulted in successful fusion in all animals (3/3). (Table 2A and B)

Characterizing LMP-1 Induced Osteogenesis using Ectopic Implants

Animals were euthanized at the prescribed dates, and the implants were removed and assessed by non-decalcified histology using both haematoxylin and eosin (H & E), or Goldner Trichrome staining.

As early as day 3, H&E histology showed migration and proliferation of cells in the implants loaded with the buffy coat cells that were transduced with the AdV5hLMP-1. This proliferation of cells continued over the 28 days, with areas of increasing organization. Implants loaded with buffy coat cells, that were transduced with AdV5βGal, showed little or no influx of host cells, and a general lack of cells throughout the study. In addition, there was an absence of cells in the central areas of all implants after day 5.

As early as day 5, sections stained with Goldner Trichrome showed the beginning of bone formation at the edges of the implants that received the cells transduced with the AdV5hLMP-1. (Figure 2) At day 7, clearly defined calcified tissue, with lacunae, was seen at the periphery, and the beginnings of ossification was evident further into the implant. (Figure 3) Histologic evidence of continued bone formation continued throughout the 28 day period in implants loaded with AdV5-hLMP-1 transduced cells. (Figure 4) Implants loaded with AdV5βGal transduced cells showed no new bone formation and little evidence of cells, corroborating the H&E results (Figure 5).

Discussion

The most important findings generated over the course of these studies are that expression of the novel intracellular LMP-1 protein consistently induced new bone formation in vivo, and that spine fusion could be achieved in an immune competent animal with the use of local gene therapy.

In addition, we established that cells readily obtained from peripheral blood were suitable vehicles for induction of new bone and spinal fusion. This approach obviates the need to either harvest autogenous bone from a second surgery site, with its associated morbidity, or to harvest marrow cells, which is

a more invasive procedure.

Prior studies, using adenoviral vectors and bone morphogenetic protein genes to induce spine fusion, have yielded inconsistent results. These studies were limited by the use of high viral doses,[11-14] protracted viral transduction times[15,16] or the need to select or expand a desired cell population.[17,18]

The studies that are summarized here showed consistent bone induction, with a very short transduction time (10 minutes) and a low viral dose (MOI = 4 to 10), in a challenging spine fusion model. Previous studies, using this same rabbit model, have shown that autogenous bone grafts achieved a successful fusion rate of only 50-70%.[19,20,21]

In addition, because of the availability of the target cell source, and the short transduction time, this protocol may be adapted to an intra-operative setting.

Although we utilize an ex vivo transduction protocol, the presence of an immune response to adenovirus is an issue, since there are still significant serum proteins present in the buffy coat cells. Pre-existent antibodies to adenoviruses are extremely prevalent in the human population. These antibodies exist in 23% of seven-month old children,[22] with the percentage nearing 100% in the adult population.[23,24] While almost all adults have total antibodies (TAb) to type 5 adenovirus, only 57% have neutralizing antibodies (NAbs).[25] Even this reduced number remains a problem, especially since we are utilizing a relatively small amount of virus.

To better understand these hurdles, we developed a model system that permits the study of the effects of human AdV5 pre-exposure on ex vivo transduction and subsequent arthrodesis. By immunizing previously naïve rabbits with AdV5βGal, we were able to establish NAbs in their serum, using a sensitive assay.

Four weeks after immunization, we were unsuccessful in our attempts to generate a spine fusion with the same cell number and viral dose that was effective in naïve animals. Even sixteen weeks post immunization, there was still a residual anti-adenovirus effect that limited fusion success to 67%.

To circumvent the immune response, individual increases in cell number, viral dose, and a combined increase in both elements were examined at a time point that was four weeks post-immunization. Increasing the viral dose from an MOI of 4 to 10, a 2.5 fold increase, had no effect. However, increasing the cell number from four to ten million

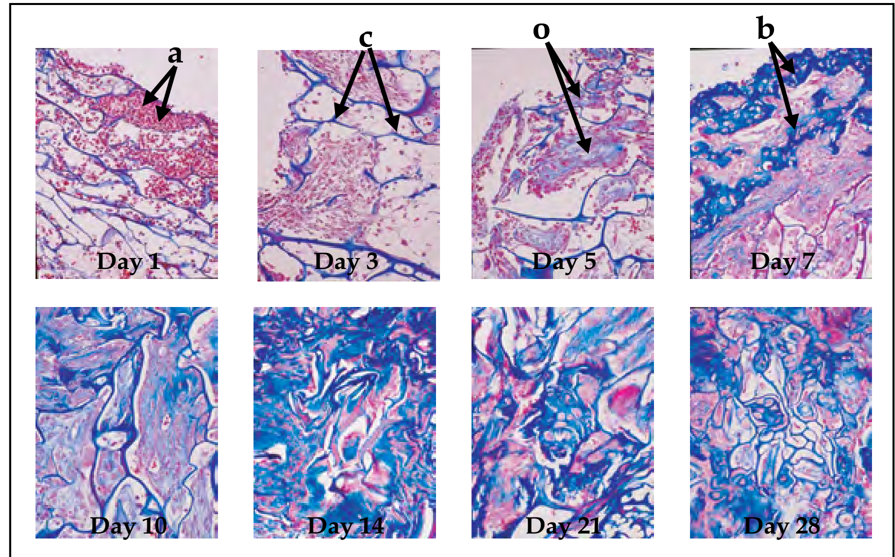

Figure 4: Time course histology with Goldner Trichrome stained sections of ectopic implants excised from athymic rats at days 1, 3, 5, 7, 10, 14, 21 and 28 post implantation. Implants were loaded with 1 million cells transduced with either AdV5hLMP-1 at an MOI = 4 for 10 minutes. These images demonstrate the de novo bone formation induced by the AdV5hLMP-1 transduced cells. Increased influx of host cells (a) towards and later into the carrier is noted as early as day 3. Collagen fibers of the carrier stain blue and are labeled (c). Bone formation (o) indicated by mineralized tissue is evident at day 5 with bony trabeculae (b) appearing at day 7. Images for day 10 through 28 are from more internal sections of the implants as the periphery were all bone as seen on day 7. There is a progressive increase in cells and bone formation moving inward from the periphery of the implants.

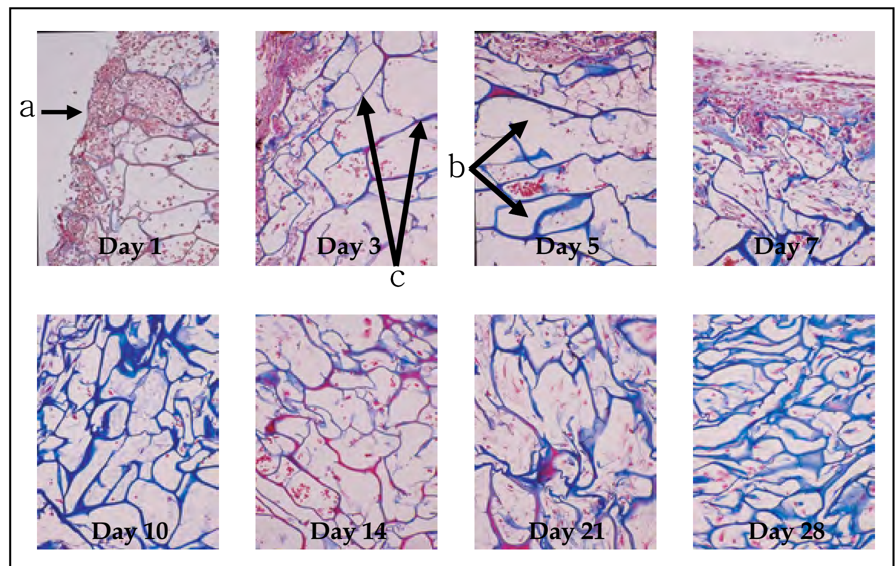

Figure 5. Time course histology with Goldner Trichrome stained sections of ectopic implants excised from athymic rats at days 1, 3, 5, 7, 10, 14, 21 and 28 post implantation. Implants were loaded with 1 million cells transduced with either AdV5βGal at an MOI = 4 for 10 minutes. These images demonstrate an absence of any bone formation induced by the AdV5βGal transduced cells. While there is an accumulation of host cells at the periphery of the implant on day 1 and 3 (a), there is neither an increase in host cell accumulation on successive dates, nor is there any host cell proliferation or migration into the carrier (b). Collagen fibers of the carrier stain blue and are labeled (c). There is no evidence of mineralized tissue indicative of bone formation present at any time during the 28 days.

overcame some of the response, yielding fusons in two out of three animals. However, if both the cell number and virus dose were increased to 10 million cells and an MOI of 10, respectively, we were able to overcome the immune response and achieve a successful fusion in all three of the rabbits.

Therefore, it appears that this immune response can be circumvented with minor modifications in the protocol.

The mechanism for de novo bone formation, induced by LMP-1, was examined over twenty-eight days, using ectopic implants in an athymic rat model. Use of these animals allowed the examination of xenogeneic blood sources including human. Buffy coat cells isolated from human peripheral blood and transduced with AdV5h-LMP-1, or a negative control AdV5βGal, were implanted in athymic rats and harvested at set times, post surgery. Histology revealed LMP-1 induced recruitment of host cells, and the proliferation of cells and bone formation, starting at the periphery of the implant. As early as day 5, bone formation was evident in Goldner Trichrome stained sections. The ossification continued an inward progression during the 28-day period. Parallel implants, loaded with AdV5βGal transduced cells, exhibited neither the recruitment nor bone induction, and the collagen implants were soft, and/or partially resorbed, at the 28-day harvest.

It is also of note that past day 5, few, if any, cells are visible in the interior of the implants. This indicates that once bone formation is induced by the LMP-1 transduced cells, it relies upon host cells. Based upon the histology, LMP-1 induced de novo bone appears to be direct membranous bone formation.

In summary, these results suggest that local gene therapy, using an adenoviral vector to deliver LMP-1 cDNA, is a feasible and promising alternative method to achieve de novo bone formation and spine fusion. In addition, the use of an ex vivo, low dose, transient gene transfer protocol should minimize any host immune response or toxicity issues that have been noted in other therapies. The short transduction times and low viral doses, coupled with an easily attained target cell population, should allow for the development of a clinically feasible intra-operative therapy.

Acknowledgments

The author wishes to acknowledge that the majority of the new research reviewed in this manuscript was performed by H. S. Kim, M.D., A. Minamide, M.D., M. Viggeswarapu, Ph.D. Y. Liu, Ph.D., G. Hair, Ph.D., C. Oliver, D.V.M. and L. Titus, Ph.D. with and under the guidance of S.D. Boden, M.D. of the Department of Orthopaedic Surgery, Emory University School of Medicine and the Atlanta Veterans Affairs Medical Center in Atlanta, GA.

References

1. DePalma, A.F. and Rothman, R.H. (1968) The nature of pseudoarthrosis, ***Clinical Orthopedics***, 59:112-118.

2. Steinman, J.C. and Herkowitz, H.N. (1992) Pseudoarthrosis of the spine. ***Clinical Orthopedics***, 284:80-90.

3. Fernyhough, J.C., Schimandle, J.H., Weigel, M.C., Edwards, C.C., and Levine, A.M. (1992) Chronic donor site pain complicating bone graft harvesting from the posterior iliac crest for spinal fusion, ***Spine***, 17: 1474-80.

4. Way, J.C. and Chalfie, M. (1988) Mec-3, a homeobox-containing gene that specifies the differentiation of the touch receptor neurons in C. elegans, ***Cell***, 45: 5-16.

5. Karlsson, O., Thor, S., Norberg, T., Ohlsson, H. and Edlund, T. (1990) Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeodomain and a Cys-His domain. ***Nature***, 344:879-882.

6. Freyd, G., Kim, S.K. and Horvitz, H.R. (1990) Novel cysteine-rich motif an homoeodomain in the product of the Caenorhabditis elegans cell lineage gene lin-II. ***Nature***, 344:876-879.

7. Boden, S.D., Liu, Y., Hair, G.A., Helms, J.A., Hu, D., Racine, M., Nanes, M.S. and Titus, L. (1998) LMP-1. A LIM-Domain protein, mediates BMP-6 effects on bone formation. ***Endocrinology***, 139:5125-5134.

8. Boden, S.D., Titus, L., Hair, G.A., Liu, Y., Viggeswarapu, M. and Nanes, M.S. (1998) Lumbar spine fusion by local gene therapy with a cDNA encoding a novel osteoinductive protein (LMP-1). ***Spine***, 23:2486-2492.

9. Mitteredere, N., March, K.L. and Trapnell, B.C. (1996) Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. ***J. Virology***, 70:7498-7509.

10. Boden, S.D., Schimandle, J.H. and Hutton, W.C. (1995) An experimental lumbar intertransverse process spinal fusion model: Radiographic, histologic and biomechanical healing characteristics. ***Spine***, 20:412-420.

11. Chirmule, N., Propert, K.J., Magosin, S.A., Qian, Y., Qian, R., and Wilson, J.M. (1999) Immune responses to adenovirus and adeno-associated virus in humans. ***Gene Therapy***, 6:1574-1583.

12. Harvey, B.G., Worgall, S., Ely, S., Leopold, P.L. and Crystal, R.G. (1999) Cellular immune responses of healthy individuals to intradermal administration of an E1-E3- adenovirus gene transfer vector. ***Human Gene Therapy,*** 10:2823-2837.

13. Brann, T., Kayda, D., Lyons, R.M., Shirley, P.,Roy, S., Kaleko, M. and Smith, T.(1999) Adenoviral vector-mediated expression of physiological levels of human factor VIII in non-human primates. ***Human Gene Therapy***, 10:2999-3011.

14. Zuckerman, J.B., Robinson, C.B., McCoy, K.S., Shell, R., Sferra, T.J., Chirmule, N., Magosin, S.A., Properet, K.J., Brown-Parrr, E.C., Hughes, J.V., Tazelaar, J., Baker, C., Goldman, M.J. and Wilson, J.M. (1999) A phase I study of adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator gene to a lung segment of individual with cystic fibrosis. ***Human Gene Therapy***, 10: 2973-2985.

15. Lieberman, J.R., Le, L.Q., Wu, L., Finerman, G.A., Berk. A., Witte, O.N., and Stevenson, S. (1998) Regional gene therapy with a BMP-2 producing murine stromal line induces heterotropic and orthotropic bone formation in rodents. ***J. of Orthopedic Research***, 16:330-339.

16. Lind, M. Deleuran, B., Thestrup-Pedersen, K., Soballe, K., Ericksen, E.F. and Bünger, C. (1995) Cheomtaxis of human osteoblasts: Effects of osteotropic growth factors. ***APMIS*** (***Acta Pathologica, Microbiologica, et Immunologica Scandinavica***), 103:140-146.

17. Lou, J., Xu, F., Merkel, K., and Manske, P. (1999) Gene therapy: Adenovirus-mediated human bone morphogenetic protein-2 gene transfer induces mesenchymal progenitor cell proliferation and differentiation in vitro and bone formation in vivo. ***J. Orthopedic Research***, 17:43-50.

18. Riew, K.D., Wright, N.M. Cheng, S.L., Avioli, L.V., and Lou, L. (1998) Induction of bone formation using a recombinant adenoviral vector carrying the human BMP-2 gene in a rabbit spinal fusion model. ***Calcified Tissue Int.***, 63:357-360.

19. Boden, S.D., Martin, G.J., Morone, M.A., Ugbo, J.L. Titus, L. and Hutton, W.C. (1999) The use of coralline hydroxyapatite with bone marrow, autogenous bone graft or osteoinductive bone protein extract for posterolateral lumbar spine fusion. ***Spine***, 24:320-327.

20. Boden, S.D., Schimandle, J.H., Hutton, W.C., Damien, C.J., Benedict, J.J., Baranowski, C. and Collier, S. (1997) In vivo evaluation of a resorbable osteoinductive composite as a graft substitute for lumbar spine fusion. ***J. Spinal Disorders***, 10:1-11.

21. Morone, M.A. and Boden, S.D. (1998) Demineralized bone matrix as a graft extender in posterolateral lumbar spine arthrodesis. ***Spine***, 23:159-167.

22. Nelson, J.K., Shields, M.D., Stewart, M.C. and Coyle, P.V. (1999) Investigation of seroprevalence of respiratory virus infections in an infant population with a multiantigen fluorescence immunoassay using heel prick blood samples collected on filter paper. ***Pediatric Research***, 45:799-802.

23. D'Ambrosio, E., Del Grosso, N., Chicca, A. and Midulla, M. (1982) Neutralizing antibodies against 33 human adenoviruses in normal children in Rome. ***The Journal of Hygiene***, (London) 89:155-161

24. Piedra, P.A., Poveda, G.A., Ramsey, B., McCoy, K. and Hiatt, P.W. (1998) Incidence and prevalence of neutralizing antibodies to the common adenoviruses in children with cystic fibrosis: Implications for gene therapy with adenovirus vectors. ***Pediatrics***, 101:1013-1019.

25. Schulick, A.H., Vassalli, G., Dunn, P.F., Dong, G., Rade, J.J., Zamarron, C., and Dichek, D.A. (1997) Established immunity precludes adenovirus-mediated gene transfer in rat carotid arteries. Potential for immunosuppression and vector engineering to overcome barriers of immunity. ***J. Clinical Investigation***, 99:209-219.