INTRODUCTION: LMP mineralization protein-1 (LMP-1), an osteoinductive protein identified in 1998, is thought to induce secretion of soluble factors that convey its osteoinductive activity. Unlike BMPs which are extracellular proteins that act through cell surface receptors, LMP-1 is thought to be an intracellular signalling molecule and thus requires gene transfer of its cDNA to have any therapeutic effect. Although evidence suggests LMP-1 to be a critical regulator of osteoblast differentiation in vitro and in vivo, little is known about its mechanism of action. Several basic questions remain unanswered: 1) Does LMP-1 induce endochondral or membranous bone formation? 2) Does LMP-1 induce bone in a centripetal pattern like BMPs or simultaneously throughout the implant? 3) How long do the transplanted cells survive in the implant? and, 4) Does LMP-1 induce expression of BMPs? The purpose of the present study was to describe the time sequence of histologic changes during bone formation induced by LMP-1 and answer the questions posed above.

METHODS: Sixteen athymic rats received four subcutaneous implants on the chest. Rabbit or human buffy coat cells from peripheral blood were used to deliver the LMP-1 cDNA. One million cells per implant were infected with recombinant (E1E3-deleted) type 5 human adenovirus (Multiplicity of Infection MOI = 4.0) for 10 minutes, placed on a type I collagen disc and implanted. The buffy coat cells contained either Ad-CMV-LMP-1 (active) or Ad-CMV-Bgal (control). The animals were sacrificed at 1, 3, 5, 7, 10, 14, 21 and 28 days after surgery and explants were analyzed by histology and immunohistochemistry.

The specimens were fixed for 24 hours in 10 % neutral buffered formalin. The specimens were prepared for undecalcified or decalcified sectioning. The specimens at 21 and 28 days after implantation were decalcified with 10 % ethylenediaminetetraacetic acid (EDTA) solution for 3 to 5 days. The specimens were dehydrated through graded strengths of ethanol and embedded in paraffin. Specimens were sectioned at a thickness of 5 µm on a microtome (Reichert Jung GmbH, Heidelberg, Germany). Sections were subjected to hematoxylin and eosin staining, Goldner’s trichrome staining, and immunohistochemical study using antibodies specific for CD-45 (DAKO Co., Carpinteria, California), BMP-4 (Santa Cruz Biotechnology, Inc., Santa Cruz, California), BMP-7 (Santa Cruz Biotechnology, Inc., Santa Cruz, California), and collagen type I (Sigma Chemical Co., Saint Louis, Missouri). The staining procedure was performed with the labeled streptavidin-biotin method (LSAB method). A kit (Universal LSAB Kit, Peroxidase; DAKO Co., Carpinteria, California) was used for immunostaining with antibodies. Endogenous peroxidase was blocked with methanol containing 0.3 % hydrogen peroxide. Specimens were incubated with phosphate buffered saline (PBS) containing either 5 % normal rabbit serum or 5 % normal goat serum, and 1 % bovine serum albumin for 15 minutes at room temperature to avoid nonspecific binding and then with a dilution of 1:100 of primary antibodies at 4 C overnight in a humidified chamber. After washing with PBS three times for 5 minutes, followed by incubation with biotinylated secondary antibody and streptavidin-biotin-peroxiadase complex in a humidified chamber for 10 minutes at room temperature, color was developed using 3,3’- diaminobenzidine tetrachloride (DAB; DAKO Co., Carpinteria, California). Finally, the sections were counterstained by hematoxylin. As negative controls, PBS, normal mouse IgG and normal goat serum was used instead of the primary antibodies.

RESULTS: By Day 3, an increased number of cells were seen at the periphery of the Ad-LMP implants. By Day 5, the number of cells surviving in the center of the implant was diminished, especially in the controls. Extracellular matrix could be seen deposited near the cells in the periphery by day 7 in the Ad-LMP implants. By Day 10, osteoblast-like cells were observed in the gaps between the collagen fibers. Osteoid and some mineralized bone were consistently seen by Day 14 growing inward from the edge of the implant. By Day 28, haversian systems were seen with osteoblasts, osteoclasts and bone marrow elements.

At one and three days after implantation, lymphocytes detected by anti-CD-45 antibody were abundantly present in buffy coat cells in both the active and control groups. These cells were part of the buffy coat cells placed in the original implant. The number of cells with the specific reaction gradually decreased after implantation. By Day 10 few cells having specific staining for anti-CD-45 remained. Immunohistochemistry revealed some strong BMP-4 and BMP-7 staining on Day 3 and Day 5 in Ad-LMP specimens within lymphocyte cells on the collagen fibers. Later (Day10-28), the positive staining for BMP-4 and -7 was more prevalent as additional osteoblastic cells were present. The strong collagen type I staining was observed in the Ad-LMP implants Day 10, 14, 21 and 28. The specific reaction corresponded to the existence of osteoblast-like cells and new bony matrix with the strong staining on the periphery of these cells and in the extracellular matrix.

DISCUSSION: Bone was induced ectopically with buffy coat cells infected with Ad-LMP-1 while no bone formed in implants containing cells infected with Ad-Bgal. LMP-1 transfected buffy coat cells induced direct intramembranous bone formation without evidence of a cartilage intermediate phase. It appears that BMP-4 and BMP-7, two candidate BMPs chosen based on in vitro mRNA studies, may be early intermediate secreted factors that can recruit and differentiate mesenchymal cells from the periphery of the implant. Although not conclusive, the loss of anti-CD-45 staining, the dropout of cells in the center of the implant by 7 days, and the centripetal pattern of bone formation all suggest that the transplanted cells expressing LMP-1 may not survive long. The LMP-expressing cells may only participate indirectly in the bone formation process through their induced secreted factors (BMPs). These studies demonstrate the histologic healing sequence of bone induced by ex vivo gene transfer of LMP-1 cDNA to peripheral buffy coat cells in an ectopic location and begin to answer some of the questions as to the mechanism of bone formation with LMP-1 at the macroscopic level.