must take place for 6-24H for inhibition to occur, suggesting that de novo synthesis of proteins is involved. MAPK is inactivated by dephosphorylation of tyrosine and threonine residues. Inhibition of tyrosine phosphatases (sodium orthovanadate), but not serine/threonine phosphatases (sodium fluoride) restored both pre-osteoblast proliferation and a normal ERK activation profile, suggesting that DMS exerts its effects by up-regulating growth-inhibitory tyrosine phosphatases. Western blotting indicated that at least two tyrosine phosphatases, SHP-1 and PTP-1B, are up-regulated by DMS. Unlike PTP-1B, SHP-1 was up-regulated in a dose and time dependent fashion and co-immunoprecipitated with ERK, suggesting that it is one of the tyrosine phosphatases involved in down-regulating ERK activity.

The effects of sodium orthovanadate was subsequently assessed in a rat model of steroid-induced osteoporosis. Four month old Sprague-Dawley rats were treated with prednisolone (3.5mg/kg/day s.c. for 9 weeks) alone or in combination with vanadate (0.5mg/ml drinking water, ad lib). Steroid-treated bones were significantly ($p<0.01$) osteopenic as measured densitometrically (DEXA, Hologic). Quantitative bone histology following double tetracycline labelling confirmed a significant decrease in osteoblast numbers and rates of bone formation, while tibial breaking strength was also significantly reduced in steroid treated animals. Treatment with vanadate prevented the densitometric, histologic and physical abnormalities induced by prednisolone. We conclude that steroid-induced osteoporosis is caused, at least in part, by up-regulation of tyrosine phosphatases and that these effects can be reversed by orthovanadate.

P255 S

PERICHONDRIAL CELLS IN LIMB BUD EXPRESS AN ACTIVATED LEUKOCYTE ADHESION MOLECULE AND HAVE THE CHARACTERISTICS OF MESENCHYMAL PROGENITORS

F. Arai*, O. Ohneda, T. Miyamoto, K. Iwamoto, T. Suda
Department of Cell Differentiation, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan

During embryonic bone formation, mesenchymal progenitors or stem cells are thought to be resided in perichondrium (which is then periosteum). However, differentiation process of mesenchymal stem cells in perichondrium to osteoblasts is still unclear. To investigate whether the cells in the perichondrial region have multipotent ability to differentiate, we isolated mononuclear adherent cells from mouse E13.0 limb bud by FACS using anti-ALCAM (CD166) monoclonal antibody. It is known that ALCAM is type I membrane protein including five Ig extracellular domains, and mediates homophilic/heterophilic adhesion with CD6. In addition, it was previously reported that ALCAM was expressed on mesenchymal stem cells.

Immunohistochemical staining revealed that the localization of the expression of ALCAM in E13.0 limb bud was restricted in perichondrium. FACS analysis showed the proportion of CD31-nagative/CD45-negative/ALCAM-positive cells was 2.8% in freshly prepared limb bud cells and 38.7% in 2 days-cultivated limb bud cells. Differentiation assay for several kinds of mesenchymal cells revealed that most of cultured ALCAM-positive cells from limb bud cells differentiated into the ALP expressed osteoblasts and oil red O-positive adipocyte. In addition, limb bud derived ALCAM-positive cells have potential to support hematopoietic cell proliferation in the presence of IL-6, IL-7, SCF, and Epo. Moreover, we have already reported that ALCAM was expressed on hematopoietic cells, bone marrow stromal cells, and endothelial cells, and homophilic adhesion of ALCAM play critical role for maintenance of ALCAM-positive hematopoietic stem cell, and growth of ALCAM-positive endothelial precursor cells. In addition, recent studies have identified a population of pluripotential stem cells, also called side-population (SP) cells, using Hoechst 33342 dye. FACS analysis showed that purified limb SP cells expressed ALCAM.

These findings suggest that ALCAM is a marker of mesenchymal stem/ progenitor cells, which were reserved in perichondrium, moreover, osteoprogenitor cells are supplied from the perichondrium during endochondral ossification.

P256 W

ZINC MAPPING IN OSTEOBLASTS AND BONE MATRIX

S. Gomez
Dept Anatomia Patologica, Faculty of Medicine, University of Cadiz, Spain

The localization and distribution of zinc ions in osteoblasts and in bone matrix were visualized using the sulfide-silver method that was applied after zinc-partial chelation procedures. Chelation procedures were based on the different Zinc bonding strength to remove sequentially zinc ions prior to sulfide-exposure. With this method labil zinc (zinc-protein complexes), mineral zinc (zinc bound to mineral), and firm zinc (metalloproteins) were stained separately. In the extracellular matrix, zinc ions were found as labil zinc in osteoid, as mineral zinc in early calcified deposits, bone mineral surfaces, and cement lines, and as firm zinc as two different pools from alkaline phosphatase and from matrix metalloproteinases. In osteoblasts, zinc ions were localized diversely within the nucleus in discrete domains and in nucleoli. Others zinc pools were also located apart in intracytoplasmic vesicles, Golgi apparatus, rough endoplasmic reticulum, plasmatic membrane, and lysosomes.

P257 T

DEMONSTRATION OF BONE FORMATION IN VIVO: A NEW MODEL TO STUDY HUMAN BONE MARROW STROMAL CELLS

K. Stenderup[1]*, C. Rosada[1], J. Justesen[1], E. F. Eriksen[1], F. Dagnaes-Hansen[2], M. Kassem[1]
[1]University Department of Endocrinology and Metabolism, Aarhus County Hospital, Denmark
[2]Department of Microbiology, University of Aarhus, Aarhus, Denmark

Marrow stromal cells (MSC) are a group of mesenchymal cells located in the bone marrow. When cultured in vitro MSCs are able to differentiate into several cell lineages including the osteoblasts. In order to understand the physiological regulation of MSC differentiation and activity, we developed a model to monitor their bone forming capacity in vivo.

Human bone marrow was aspirated from iliac crest from normal human volunteers. MSCs were cultured in vitro until confluence. They were trypsinized, mixed with hydroxyapatite-tricalciumphosphate (HA/TCP) (Zimmer) and implanted s.c. in immunodeficient mice (NOD/LtSz-SCID). As negative control we used human breast fibroblasts (hBF) and HA/TCP alone. After 8 weeks, implants were removed and embedded undecalcified in methyl-methacrylate (MMA). Sections were stained histochemically with Goldner-Trichrome. In order to prove that the bone formed was of human origin, immunostaining was performed using human-specific antibodies against osteonectin and collagen type I. We also immunostained for osteocalcin and smooth muscle cells. The amount of bone was quantified by point-counting.

All the implants tested showed evidence of newly formed bone. Areas of osteoid, lamellar and woven bone were easily identified. No bone was formed in implants of hBF and in implants consisting of HA/TCP without cells. Also, the bone matrix stained positive for osteonectin and collagen type I and these antibodies exhibited negative staining of mouse bone. The amount of bone formed was positively correlated to the number of MSCs implanted (0.1×10^5 to 30×10^5 MSCs per implant) with maximal percentage of bone formation of 13% with 5×10^5 MSC per implant.

Thus, our model allows identification and quantification of MSC bone forming capacity and can be employed to understand changes of MSC bone forming capacity under physiological (e.g. aging) and pathological (e.g. osteoporosis) conditions.

P258 S

EFFECT OF OVARIECTOMY ON DEXAMETHASONE- AND PROGESTERONE-DEPENDENT OSTEOPROGENITORS IN CELL POPULATIONS DERIVED FROM VERTEBRAE AND PROXIMAL FEMURS OF FEMALE RATS

W. Pei*, C. G. Bellows, E. S. Elsubeihi, J. N. M. Heersche
Faculty of Dentistry, University of Toronto, Canada

Decreased production of ovarian sex hormones frequently leads to post-menopausal osteoporosis. Ovariectomized (OVX) rats are often used as a model to study postmenopausal osteoporosis, since this model shares many similarities with the disease in humans. In previous experiments in rats, we have found that two types of osteoprogenitors could be identified in the skeleton of adult female rats: dexamethasone (Dex)- and progesterone (Prog)- dependent osteoprogenitors. The purpose of the present study was to determine whether OVX affected the Dex- and Prog- dependent classes of osteoprogenitors differently. 6-month-old Sprague-Dawley rats were OVX and the lumbar vertebrae and proximal femurs collected 1.5, 3 and 6 months after OVX. Cells were obtained from outgrowths of explants and grown in alpha-MEM, 10% FBS, 50mg/ml ascorbic acid and 5 mM beta-glycerophosphate with or without Dex (1-100 nM) or Prog (1-10 microM) and in the presence or absence of 10 nM estrogen. Osteoprogenitors were quantitated by their ability to generate a colony of osteoblastic cells forming bone (bone nodule). In cell populations derived from the vertebrae of rats OVX for 1.5, 3 and 6 months and of control rats, both Dex and Prog dose-dependently stimulated nodule formation with maximal effect at 10 nM Dex and 10 microM Prog. Dex and Prog increased nodule formation to a greater extent in cell populations derived from control rats than from OVX rats ($p<0.001$). Estrogen enhanced the Prog-responsiveness (1-10 microM) in vertebral populations derived from both OVX and control rats, however, estrogen decreased the response in both groups to Dex (10 nM). In femoral populations from both OVX and control rats, the response to Dex (10 nM), Prog (3 microM), and the effect of estrogen (10 nM) on the response to Prog (3 microM) were similar. These results demonstrate that Prog- dependent osteoprogenitors persist in OVX rats and that Dex and Prog stimulate osteoprogenitor proliferation and differentiation in populations from both control and OVX rats. Although the number of both Dex- and Prog- dependent osteoprogenitors is decreased after OVX, the different responses to estrogen by the Dex-dependent and Prog-dependent osteoprogenitors further confirm that they constitute two distinct classes of osteoprogenitors.