TIME-DEPENDENT VEGF EXPRESSION MODULATES ECTOPIC BONE FORMATION MEDIATED BY MUSCLE-DERIVED STEM CELLS

*Usas, A; *Ho, A; *Peng, H; *Gearhart, B; **Huard, J
**Stem Cell Research Center, Children’s Hospital of Pittsburgh and Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA
jhuard@pitt.edu

INTRODUCTION:
Previous work by our group has demonstrated synergy between angiogenic and osteogenic factors in bone formation, indicating that the former is very important for endochondral ossification.1 We also noticed that excessive amounts of VEGF in relation to BMP4 impair bone healing. Therefore, tight regulation of growth factor expression may be necessary for successful bone tissue engineering. The effects of timely induced angiogenesis on bone regeneration have not been determined. Here we hypothesized that time-dependent VEGF upregulation could affect bone formation mediated by muscle-derived stem cells (MDSCs) expressing bone morphogenetic protein 4 (BMP4).

METHODS:
We constructed a self-inactivating tet-on retroviral vector expressing VEGF under the control of a tetracycline-inducible promoter.1,2 MDSCs were isolated and transduced as described previously.3 The amount of protein secreted by BMP4-transduced cells (B4) was detected using BMP bioassay.4 ELISA was used to confirm VEGF expression by MDSCs transduced with conventional (VE) or inducible retrovirus (TVE). TVE cells were exposed to different doses of doxycycline (Dox; 0, 1, and 2 µg/mL), and the amount of VEGF in conditioned medium was measured over 2, 4, and 6 days. Based on in vitro results, a normalized number of B4 and TVE cells (B4+TVE) was seeded onto Gelfoam scaffolds and implanted into the right thigh muscles of normal mice. Control mice received scaffolds containing B4 and VE cells (B4+VE). Left thigh muscles of all mice received scaffolds containing B4 cells only (B4). The 25 animals then were divided into 5 groups depending on whether or not they received Dox-supplemented drinking water and when Dox was administered after surgery. Mice in group 1 (control) and group 2 received no Dox. Dox administration began on day 0 after surgery in group 3, on day 3 in group 4, and on day 7 in group 5, and continued until scheduled euthanization. Ectopic bone formation at the implantation sites was monitored radiographically and histologically at 3 and 6 weeks post-surgery. Northern Eclipse imaging software was used to quantify ectopic bone area and bone photodensity. Cryosections of the specimens harvested at 3 weeks were stained for Von Kossa to detect mineralized bone deposition. At the end of the experiment at 6 weeks we isolated bony nodules from the muscles of remaining mice and measured their dry weight.

RESULTS:
VEGF secretion in vitro: ELISA confirmed that in the absence of Dox TVE cells secreted almost undetectable or very low levels of VEGF over the period of 6 days. Stimulated with 2 µg/mL of Dox for 2, 4, or 6 days TVE cells secreted nearly constant amount of VEGF (7 to 8 ng/ml per million cells/24h) which was 4 times less than the amount of VEGF secreted by VE cells (34 ng/ml per million cells/24h)(data not shown). Radiographic and histological examination of heterotopic bone: No difference in bone formation at any time was noticed between groups treated with B4 cells alone (Fig.1). Control mice demonstrated exorbitant ossification at B4+VE cell implantation sites. There was no apparent difference in bone formation between B4 cell and B4+TVE cell implantation sites without Dox treatment. Larger ectopic bone was evident in Dox-treated animals at B4+TVE implantation sites compared to B4 implantation sites any time VEGF expression was induced by Dox administration (Fig.1). Radiomorphometry showed larger bone area at B4+TVE implantation sites in Dox-treated mice than in nontreated mice. There was a significant increase in bone area and density after 6 weeks when Dox was administered at times other than immediately after surgery (Fig.1). Quantification of ectopic bone by weight: Bone harvested from B4+TVE implantation sites of Dox-treated animals weighed more than bone from contralateral B4 implantation sites, but the increase was significant only when VEGF expression was induced on day 3 after surgery. VEGF induction time had no significant effect on the ectopic bone weight in Dox-treated mice (data not shown).

DISCUSSION:
Our study shows that controlled VEGF expression is achievable using an inducible tet-on retroviral vector. This vector enables tight regulation of growth factor secretion by transduced MDSCs. Dox-induced TVE cells secreted constant amounts of VEGF in vitro and enhanced ectopic bone formation mediated by BMP4-expressing MDSCs in vivo. We were able to modulate bone formation by altering the induction time of VEGF expression. Radiomorphometry and measurement of bone weight performed 6 weeks after implantation of cell-seeded scaffolds revealed the presence of larger, denser and heavier bony masses when neovascularization was triggered not immediately after the surgery, but 3 or 7 days later. This finding indicates that proper timing of VEGF expression is crucial for bone regeneration. Our study has important implications for the development of novel bone tissue engineering strategies based on angiogenic gene regulation.

ACKNOWLEDGEMENTS:
We wish to thank Jim Cummins for his editorial assistance. This work was supported by a grant to Dr. Johnny Huard from the National Institutes of Health (NIH R01-DE 13420).

REFERENCES:

Poster No: 1463