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NITRIC OXIDE SYNTHASE GENE THERAPY IN FRACTURE HEALING - A NOVEL EXPLANT CULTURE STUDY

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Introduction: Nitric Oxide (NO) is generated by three nitric oxide synthases (iNOS, eNOS and bNOS). Inducible iNOS produces larger amount of NO than that produced by constitutively expressed eNOS and bNOS. We previously reported [1] that nitric oxide modulates fracture healing. Inhibition of NOS inhibited the healing process; addition of NO to the fracture site reversed this impairment. We also demonstrated [2] that the expression of NOS isoforms during fracture healing is time-dependent, isoform-specific and cellular distinctive. In order to investigate the mechanisms by which NO may effect fracture healing, NO was delivered via adenovirus' carrying either human iNOS gene or human eNOS gene in a novel rat-fracture-callus explant culture gene therapy model and the mRNA expression of alkaline phosphatase (ALP), osteocalcin (OC), basic fibroblast growth factor (bFGF), and transforming growth factor beta (TGF-beta) was studied.

Materials and Methods: Fracture was created by three point bending in right femur of Sprauge Dawley rats (n=15). Using day-10 fracture callus, an explant culture was established in 24-well plates in triplicate. Callus tissue were divided into five groups: experimental groups were transfected with either human iNOS or human eNOS gene carried by an adenovirus vector at 10^{7} pfu/ul, control groups were either incubated with NOS inhibitor NMMA (negative control) or with NO donor SNAP (positive control) and one group of callus was incubated in unconditioned medium (baseline control). Rt- PCR and western blot were used to detect the human iNOS and eNOS gene expression in rat callus at both transcriptional and translational levels, after 3 hours and 24 hours transfection. Competitive PCR [2] reactions were used to quantitatively analyse the gene expression of ALP, OC, bFGF and TGF-beta in each group. Statistical significance were accepted at $p < 0.05$.

Results: Both human iNOS and human eNOS gene delivered by adenovirus were detected in rat fracture callus at the mRNA level (Fig1) and protein level (Fig2), after 3 hours and 24 hours transfection. There was no human iNOS or human eNOS mRNA and protein present in the non-transfected control groups.

The competitive PCR study showed that ALP, OC, bFGF and TGF-beta gene were expressed in rat fracture callus.

ALP gene (Fig3) and bFGF gene (Fig4) expression were found decreased to 50 % and 25 % of control baseline level in NOS inhibitor NMMA treated callus group, respectively. However, they were found increased in NO donor SNAP treated group, rising 1.5 fold (ALP gene) and 2 fold (bFGF gene) of baseline control level. mRNA for ALP was also found increased 30% in human eNOS transfected callus group, and mRNA for bFGF was found increased 3 fold in human iNOS transfected group, after 24 hours transfection.

OC gene (Fig5) and TGF-beta gene (Fig6) expression were found increased in NOS inhibitor NMMA treated group, rising 1.3 fold and 1.5 fold of control baseline level. However, mRNA for OC and TGF-beta were found decreased to 60 % and 45 % of control baseline level in NO donor SNAP treated group, respectively. Both OC gene and TGF-beta gene were also found decreased in human iNOS and human eNOS transfected groups, after 24 hours transfection.

Discussion and Conclusion: Our work has established a novel method for gene therapy in fracture healing. We found that human NOS gene was expressed in rat fracture callus at mRNA and protein levels. Nitric oxide up-regulated the ALP and bFGF gene expression, however nitric oxide down-regulated the OC and TGF-beta gene expression in rat fracture healing.

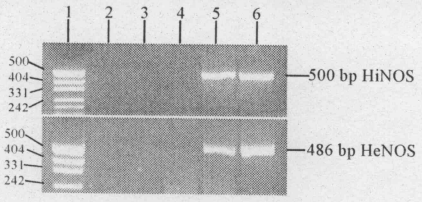

Fig1. Human iNOS (HiNOS) and human eNOS (HeNOS) cDNA expression in rat fracture callus. Lane 1: DNA marker pUC19. Lane 2: baseline control callus. Lane 3: callus treated with NOS inhibitor NMMA. Lane 4: callus treated with NO donor SNAP. Lane 5: callus transfected with either HiNOS or HeNOS for 3 hours. Lane 6: callus transfected with either HiNOS or HeNOS for 24 hours.

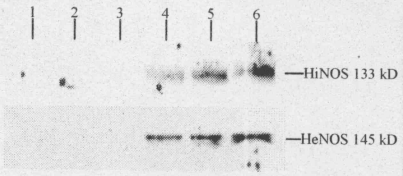

Fig2. Human iNOS (HiNOS) and human eNOS (HeNOS) protein expression in rat fracture callus. Lane 1: baseline control callus. Lane 2: callus treated with NOS inhibitor NMMA. Lane 3: callus treated with NO donor SNAP. Lane 4: callus transfected with either HiNOS or HeNOS for 3 hours. Lane 5: callus transfected with either HiNOS or HeNOS for 24 hours. Lane 6: HiNOS positive control (stimulated human chondrocytes) or HeNOS positive control (human endothelial cells).

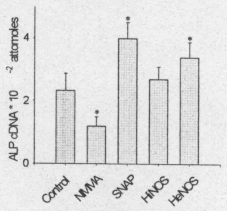

Fig3. ALP expression in 24 hours rat fracture callus explant culture.

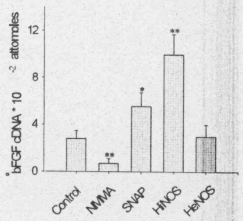

Fig4. bFGF expression in 24 hours rat fracture callus explant culture.

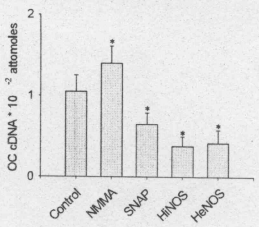

Fig5. OC expression in 24 hours rat fracture callus explant culture.

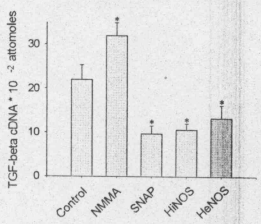

Fig6. TGF-beta expression in 24 hours rat fracture callus explant culture.

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