INTRODUCTION

It is well documented in the orthopedic literature that cigarette smoking has a negative impact on healing following long bone fracture and spinal fusion surgery. While the morbidity and financial burden of this clinical problem is significant, little progress has been made toward elucidating the underlying mechanisms that mediate these effects of smoking on skeletal healing. To begin addressing the question of mechanism, we have proposed a novel hypothesis which predicts that one of the components of cigarette smoke, nicotine, affects the healing process by interacting with the nicotinic acetylcholine receptor (nACHR) expressed on mesenchymal stem cell membranes. More specifically, we hypothesize that activation of the nACHR in stem cells that are recruited to participate in the healing process alters chondrogenic commitment and subsequent progression of the cells to terminal maturation. We predict that the net impact of nicotine on these processes will be to perturb the healing process causing a delay or abrogation of successful union. To begin testing this hypothesis, we performed in vitro assessment of signaling and phenotype in mesenchymal stem cells treated with nicotine and then we performed an in vivo femur fracture healing study in mice dosed with nicotine to mimic the exposure situation seen in the human smoking population.

METHODS

In vitro study of mesenchymal stem cells. Limb bud mesenchymal stem cells (MSCs) were derived from stage E11.5 mouse embryos and plated as a monolayer in 12 well plates (2x10^5 cells per well), or as a micromass in 24 well plates or on glass coverslips (1x10^5 cells/micromass, 1 micromass per well or per coverslip). mRNA Expression of various nACHR subunits was evaluated via real time RT-PCR and nicotine-induced second messenger signaling was measured via fluorescence microscopy to detect Ca^2+ transients and via luciferase assay using the cyclic AMP response element promoter luciferase reporter (CREB-luc). Phenotypic effects of nicotine were assessed in the MSC micromass cultures by measuring the expression of the chondrocyte hallmarks type 2 and type X collagen via real time RT-PCR and by assessing nodule formation via alcian blue staining of the cultures and quantitation of nodule area via histomorphometry.

In vivo assessment of femur fracture healing. Alzet minipumps (model 2004) were employed to deliver nicotine or 0.9% NaCl to mice over a 4 week period. Pumps were implanted intraperitoneally and were calibrated to deliver a continuous dose of nicotine that was analagous to the human blood nicotine levels seen in smoker consuming 20 cigarettes per day. Closed, stabilized femur fractures were created in nicotine dosed and control mice using the Einhorn device 3 days after pump implantation. Fractured femurs to be assessed histologically were harvested at 7, 14, 21 and 28 days, fixed in formalin, decalcified and embedded in paraffin. Three micron thick sections were cut and stained with hematoxylin and alcian blue. Cartilage area within the calluses were comprised of more than 60% woven bone, compared to only 15% woven bone in the treated animals. By 21 and 28 days, the effect of nicotine became negligible, with the callus comprised of >90% woven bone by 21 days for both groups. Consistent with this, mRNA extracted from calluses revealed enhanced expression of type 2 collagen at days 7 and 14 and a reduced level of type X collagen expression at these same time points in the nicotine-dosed mice. As expected, nACHR subunits, in particular α4, α5, β2 and γ, were expressed in the callus, with maximum expression being early (day 7) followed by a rapid decrease of the transcripts to undetectable levels by day 21. Overall, these interesting findings establish that nACHR subunits are expressed at the site of fracture repair and suggest that at early time points, nicotine alters progression of healing by inducing abnormal accretion of cartilage and delaying conversion of that cartilage into woven bone.

RESULTS

MSCs express α4, α5, β2 and γ nACHR subunits and propagate second messenger signals in response to nicotine. A comprehensive evaluation of nACHR expression in MSCs was performed. Micromass cultures, which spontaneously undergo chondrogenesis and chondrocyte maturation, were plated and mRNA was harvested at day 1, 3, 5 and 7. Of the 15 possible nACHR genes, only α4, α5, β2 and γ subunits were expressed in MSCs. Their expression was maximal at day 1 in culture and progressively decreased as the cells completed chondrogenesis and began to hypertrophy, an event marked by an increase in type X collagen expression. By day 7, the nACHR transcripts were lost. We next assessed if the expression of these transcripts could lead to the assembly of a signaling-competent receptor that could act as a Ca^2+ channel and also induce CREB signaling. Fluorescence microscopy with the Ca^2+ sensitive dye fura-2 showed a robust influx of Ca^2+ following treatment of the cells with doses of nicotine as low as 0.1 μM. These Ca^2+ responses could be antagonized with mecamylamine, a nACHR inhibitor with broad specificity. In addition to Ca^2+ responses, nicotine induced a dose-dependent increase in CREB signaling, evidenced by a 5-fold induction of the CREB-luc promoter/reporter. These data collectively indicate that MSCs express receptor subunits that assemble into a complex competent to transduce signaling events...

Nicotine enhances cartilage deposition but delays callus remodeling during femur fracture healing in the mouse

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DISCUSSION

The deleterious effect of cigarette smoke on the duration of healing and union rates following long bone fracture and spinal fusion surgery represents an important clinical dilemma. The morbidity and financial burden of this problem is significant, yet little progress has been made toward elucidating the underlying mechanisms that facilitate these negative effects of smoking. Findings presented here include the identification of nACHR subunit mRNAs in MSCs that lead to the formation of a complex that is competent to transduce second messenger signals. These signals likely participate in the accelerated chondrogenesis and delayed chondrocyte hypertrophy seen in nicotine-treated MSC cultures. Furthermore, we show evidence for an analogous effect of nicotine in vivo. Nicotine-dosed mice show rapid accretion of cartilage in callus formed around femur fractures that persists beyond what is seen in the saline-exposed control group. Also, mRNA assessment of callus from nicotine-exposed mice suggests enhanced type 2 collagen expression and delayed/blunted type X collagen expression. In conclusion, based on these findings, we suggest that nicotine affects skeletal healing i) by accelerating stem cell commitment to chondrogenesis and ii) by subsequently inhibiting chondrocyte differentiation toward hypertrophy.