SMOKING CAUSES DECREASED CELLULAR DENSITY AND DECREASED TYPE I COLLAGEN GENE EXPRESSION IN A MOUSE MODEL OF MEDIAL COLLATERAL LIGAMENT HEALING

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INTRODUCTION
Ligament injuries account for a significant percentage of the musculoskeletal injuries treated by Orthopaedic Surgeons [1]. The large number of MCL injuries each year lead to a significant amount of morbidity with an associated cost to society in terms of lost work productivity and recreational ability. The morbidity associated with MCL injury may be increased by exposure to cigarette smoke, as it is in other orthopaedic conditions such as hip fracture, osteomyelitis, and fracture [2-3]. Currently, the cellular and molecular basis for the deleterious effect of smoking on wound healing is poorly understood. A better understanding of this mechanism would lead to strategies to abrogate the deleterious effect of smoking on healing. In the current study, a murine model of MCL healing was used to test the hypothesis that smoking impairs extracellular matrix synthesis after injury.

METHODS
All experiments were performed under the approval of the Animal Care Committee of Washington University School of Medicine. Forty skeletally mature, male, 5 month old 129X1-SVJ mice were divided into two groups, a nonsmoking control group and a group exposed to smoke for 2 months prior to surgical MCL injury using the standard protocol of Washington University [4]. MCL injury was performed using a 27ga needle to induce blunt rupture of the ligament 2mm distal to the joint line. Mice were euthanized at 3 and 7 days after surgery. Hematoxylin and eosin staining defined structural anatomy at the site of injury. Propidium iodine staining of cell nuclei enabled quantification of cellular density of injured and sham ligaments. Immunohistochemical reactivity for proliferating cell nuclear antigen (PCNA) and the p20 subunit of caspase-3 detected proliferation and apoptosis respectively. In situ hybridization for the alpha 1 chain of type I collagen was used as a marker for type I collagen gene expression during the healing process.

RESULTS
Propidium Iodine Staining
In the normal healing process, there is no change in the cell density of the injured MCL at 3 days after injury, but a marked increase in cell density by 7 days after injury (Fig. 1). In mice exposed to cigarette smoke, there is a slightly higher cell density compared to controls at three days after injury (p = 0.03). The dramatic increase in density of the injured MCL seen between day 3 and day 7 during the normal healing process is not present in the mice exposed to smoke. The cell density of the injured control MCL is 36% higher at day 7 compared to density of the injured MCL in smokers (p = 0.01).

In situ hybridization for the alpha 1 chain of type I collagen
At day 3, there was very little gene expression in either mice exposed to cigarette smoke or the controls (Fig. 2C). At day 7, gene expression was higher in both groups compared to the day 3 time point. While both groups showed an increase in collagen gene expression from day 3 to day 7, the increase in the controls was much more pronounced than the increase in mice exposed to cigarette smoke (Fig 2A-C). At day 7, many more spindle-shaped, darkly stained cells were seen in the body of the injured control ligaments compared to smoker ligaments (Fig 2D). Since fibroblasts are spindle-shaped cells and the major producer of type I collagen in ligaments, it appears as though there are more fibroblasts producing collagen in the injured MCL of the controls compared to the smoking mice at day 7.

Figure 1: Cellular density of the medial collateral ligament of mice between smokers and nonsmoking controls at day 3 and day 7.

Proliferating Cell Nuclear Antigen (PCNA) Staining
The level of PCNA staining at the site of injury was low in both smokers and controls. When PCNA staining was present, it was seen in the tibial periosteum and granulation surrounding the site of MCL injury, but it was only rarely seen in the body of the injured MCL. No difference in proliferation was observed between the two groups.

Immunohistochemistry for Caspase-3
There was no specific reactivity for caspase-3 at the site of MCL injury in either the smokers or controls at day 3 or day 7.

DISCUSSION
In this study, we demonstrated for the first time that cellular density in an injured MCL increases dramatically between three and seven days after injury in normal wound healing. Additionally, we showed that this increase in density is inhibited in mice exposed to cigarette smoke. This is also the first study to assess cellular and molecular mechanisms of smoking on ligament healing in mice. We found that neither proliferation nor apoptotic cell death accounts for the differences in cell density seen between the two groups in our animal model. Consequently, it is likely that recruitment of cells to the site of injury is the cause of the difference seen in cellularity between smokers and controls in the current study. In addition to decreased cell density after injury in mice exposed to cigarette smoke compared to controls, we found that smokers demonstrated impaired or delayed functional healing as evidenced by lower type I collagen gene expression at 7 days after injury. The results of this study suggest that smoking cessation may be indicated after MCL injury to prevent increased morbidity associated with impaired or delayed healing caused by exposure to cigarette smoke.

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REFERENCES