INTRODUCTION:
Herniated discs (HDs) are a common disease responsible for symptoms in up to 40% of all patients with lower back pain. We should elucidate the precise mechanism of HD resorption and vascularization, which will result in the establishment of novel diagnostics and therapeutics for lower back pain.

We previously demonstrated that VEGF (vascular endothelial growth factor) and its receptors were expressed in human herniated disc (HD). TNF-α induced VEGF, resulting in neovascularization of disc tissues in a model of the acute phase of HD derived from murine macrophages and disc tissues. The goal of the current research was to investigate the precise role of TNF-α-induced VEGF and the mechanism of angiogenesis in disc tissues.

We performed ELISA, western blots, and immunohistological examinations and in vitro angiogenesis assay to assess the role of TNF-α-induced VEGF using organ disc culture. Our data highlight that angiogenesis activity in disc tissues is regulated by VEGF and the NF-kappaB pathway, which are induced by TNF-α. The angiogenic activity in disc tissues closely related to aging. Thus, prognosis of HD and rate of the resorption process of HD in patients may be different depending on patient’s age, because neovascularization of HD is indispensable for the HD resorption.

METHODS:
Coccygeal intervertebral disc tissues were obtained using a dissecting microscope after skin and soft tissues were removed. Whole intervertebral disc tissues were cultured in the absence or presence of recombinant mouse (rm) TNF-α. For NF-kappaB inhibition experiments, 1µM IMD0354 or 10µM MG132 as NF-kappaB inhibitors, and 10µM PD98059 or SP600125 as mitogen-activated protein kinase (MAPK) inhibitor was administered to disc culture medium before TNF-α stimulation. Culture media were clarified by centrifugation and processed for determination of VEGF by ELISA (n=3). Murine organ culture disc tissues were used for immunohistological staining of VEGF.

To elucidate sequential changes in VEGF expression in murine disc tissues related to aging, we investigated VEGF protein expression by ELISA and immunohistological examination in disc tissues harvested from mice of varying ages (2–32 weeks). To assess angiogenic activity as a function of age of murine intervertebral discs, we used an angiogenesis assay that can monitor new blood vessel formation and visualize vascular tubule formation.

RESULTS:
We compare various inflammatory cytokines such as IL-1β and IFN-γ, and LPS in order to induce VEGF in disc tissues. VEGF expression was significantly increased only in the presence of 10 ng/ml rm TNF-α by ELISA. To clarify the intracellular signaling pathways involved in TNF-α-induced VEGF production in murine disc cells, we also examined the effects of several inhibitors, including IMD0354 and MG132 as inhibitors of NF-kappaB, PD98059 and SP600125 as inhibitors of MAPK. The NF-kappaB pathway inhibitors, IMD0354 and MG132, significantly suppressed TNF-α-induced VEGF production. VEGF protein expression in disc tissue culture supernatants gradually diminished as age increased, from 2-week-old mice to 32-week-old mice. Immunohistological analysis with anti-VEGF antibody revealed that VEGF immunoreactivity was detectable predominantly in annulus fibrosus cells compared with nucleus pulposus cells in murine disc tissues. In addition, VEGF-positive cells were abundant in rm TNF-α-stimulated disc tissues compared with untreated samples. VEGF-positive cells were suppressed with increasing age, from 2 week-old mice to 32-week-old mice, in annulus fibrosus disc tissues. By angiogenesis assay, although CD31-positive endothelial cells and anastomosing tubule network formation were abundant in culture medium derived from 2-week-old murine disc tissues, modest tubule formation was observed in medium from 32-week-old murine disc tissues. In addition, CD31-positive cells were significantly decreased when in the presence of the NF-kappaB pathway inhibitor IMD0354.

DISCUSSION:
Our previous report indicated that the potent angiogenic factor VEGF may play an important role in the resorption process in HD. We demonstrated that TNF-α promotes the generation of VEGF in disc tissues in both a time- and dose-dependent manner. TNF-α strongly up-regulated VEGF production in disc cells compared with other inflammatory stimuli, including IL-1β, IFN-γ, or LPS. Taken together, NF-kappaB pathway inhibitors strongly suppressed VEGF induction compared with MAPK pathway inhibitors. VEGF immunoreactivity was detected predominantly in annulus fibrosus cells compared with nucleus pulposus cells in murine disc tissues. Our statistical analysis revealed that VEGF-positive cells were increased after TNF-α stimulation. Both CD31 (PECAM-1)-positive endothelial immunoreactivity and anastomosing tubule network formation were elicited by the culture medium derived from disc tissues stimulated with TNF-α, whereas angiogenic activity was strongly inhibited in the presence of IMD0354 or an anti-VEGF antibody. Thus, angiogenesis factors derived from disc tissues are strongly regulated by VEGF and dependent on NF-kappaB signaling.

Interestingly, VEGF expression was gradually suppressed as the age of the mice increased from 2 weeks to 32 weeks, although TNF-α stimulation induced even disc tissues from 32-week-old mice to secrete VEGF. This result was further confirmed by our immunohistological analysis and angiogenesis assay. Our immunohistological analysis showed that VEGF-positive cells were decreased in number in disc tissues harvested from older mice. There was a concordant decrease in annulus fibrosus cells in number in relation to age. CD31 expression on endothelial cells and anastomosing tubule formation were both decreased with age. Taken together, our data suggest that VEGF-induced angiogenic activity initiated by TNF-α could be suppressed by aging. Our data suggest that the resorption process observed in HD may be less opportunity in older HD patients.