INTRODUCTION:
Despite the wealth of literature on the subject, the exact pathogenesis of nontraumatic osteonecrosis (ON) remains uncertain. It has been suggested that decreased replication capacity of mesenchymal stem cells (MSCs) or decreased MSCs activity in the bone marrow is related to nontraumatic ON (1). However, little is known about differentiation ability of MSCs according to the risk factor (alcohol-induced, idiopathic and steroid-induced) of nontraumatic ON. We hypothesize that differentiation abnormalities in MSCs of the bone marrow of the proximal femurs might be related to nontraumatic ON of the femoral head. The purpose of this study was to investigate the osteogenic and adipogenic differentiation ability of MSCs in patients with nontraumatic ON of the femoral head.

METHODS:
Ten consecutive patients with hip OA and 37 consecutive patients with nontraumatic ON of the femoral head were included in this study. A total of 37 patients with nontraumatic ON were divided into three groups: 15 patients in whom the ON was related to evident alcohol abuse, 12 in whom the ON was considered to be idiopathic, and 10 in whom the ON was related to corticosteroid therapy. For alcohol-induced osteonecrosis of the femoral head to develop in adults, the alcohol exposure threshold needs to be approximately 150 L of 100% ethanol at a weekly consumption of 400 mL or more of absolute ethanol (2). For steroid-induced ON of the femoral head to develop, the steroid exposure threshold is approximately 2000 mg prednisolone or its equivalent (2). After written informed consent was obtained from patients, bone marrow samples (5 mL) were procured from the proximal end of femur while inserting the tapered awl into the femoral canal during hip replacement surgery.

The OA group consisted of 10 patients (6 women and 4 men) with a mean age of 56 years (range, 30 to 76 years). The alcohol-induced ON group consisted of 15 patients (4 women and 11 men) with a mean age of 51 years (range, 42 to 69 years). The idiopathic ON group consisted of 12 patients (8 women and 4 men) with a mean age of 52 years (range, 31 to 69 years). The lesion was categorized as being idiopathic when there were no risk factors that are commonly associated with ON, such as alcoholism, corticosteroid therapy, radiation therapy, an injury of the hip, or abnormal steroid metabolism. The steroid-induced ON group consisted of 10 patients (7 women and 3 men) with a mean age of 45 years (range, 23 to 65 years).

Culture expansion of MSCs
Mononuclear cells from the bone marrow were separated by centrifugation in a Ficoll-Hypaque gradient, suspended in α-modified essential medium (α-MEM) containing 10% fetal bovine serum and seeded at a concentration of 1 x 10^5 cells/cm². The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. When the monolayer of adherent cells had reached 80% confluence, the cells were trypsinized, resuspended in α-MEM containing 10% fetal bovine serum and subcultured at a concentration of 1 x 10^5 cells/cm². The cells were expanded in vitro by successive subcultures, and cells between 3rd and 5th passages were used for the experiments described.

Osteogenic and adipogenic differentiation of MSCs

After plating cells to a 12 well plate (50,000 cells/cm²), the cells were grown to confluency. Then, osteogenic differentiation was induced by culturing the MSCs of confluent state for 2 weeks in an osteogenic medium and the level of extracellular matrix calcification was examined by alizarin red S staining. Osteogenic differentiation was quantified by measurement of area stained with alizarin red S by using an image analysis program. Measurements were done in duplicate at each experiment and experiments were repeated from 3 to 4 times.

To measure the alkaline phosphatase activity, the cells were washed twice with Tris-buffered saline, lifted off the plates with a scraper, and lysed by sonication. The cell lysates were reacted at 37°C for 1 hour in a buffer containing 0.1 mol/L 2-amino-2-methyl-1,3-propanediol and 5 mmol/L MgCl₂, with 10 µmol/L p-nitrophenolphosphate serving as the substrate. The alkaline phosphatase activity was expressed in terms of the mol p-nitrophenol/min/mg protein. The protein content was determined using a protein assay kit with bovine serum albumin as the standard. After plating cells to a 12 well plate (50,000 cells/cm²), adipogenic differentiation was induced by culturing the MSCs for 2 weeks in an adipogenic medium and assessed using an Oil red O staining as an indicator of intracellular lipid accumulation.

RESULTS:

Osteogenic differentiation ability of MSCs

The pattern of surface marker expression in bone marrow stromal cells was reported in our previous study (3). We used 3rd to 5th passages of bone marrow stromal cells, which maintain similar expression pattern of surface markers.

The mean percentages of the area stained with alizarin red S in patients with hip OA and whole ON were 77.8 ± 10.9% and 61.2 ± 24.0%, respectively. The osteogenic differentiation ability of MSCs in patients with whole ON was decreased compared with that in patients with OA (p < 0.05). The mean percentages in patients with alcohol-induced and idiopathic ON were found to be decreased to 52.2 ± 21.8% and 51.0 ± 19.9%, respectively. In osteogenic differentiation was statistically significant between patients with alcohol-induced ON or between patients with idiopathic ON and patients with OA (p < 0.05 and p < 0.05, respectively). For patients with steroid-induced ON, the mean percentage of the stained area was increased to 87.0 ± 8.1% compared with patients with OA but the difference was not significant (p > 0.05). When the mean percentages from the ON patient groups were compared, there was a significant difference between patients with steroid-induced ON and those with alcohol-induced or idiopathic ON (p = 0.05 and p < 0.05, respectively). The difference between alcohol-induced ON and idiopathic ON was not significant (p > 0.05).

The mean alkaline phosphatase activities in patients with hip OA and whole ON were 13.56 ± 2.12 and 10.29 ± 2.95 mol p-nitrophenol/min/mg protein, respectively. The difference in alkaline phosphatase activities was statistically significant between patients with hip OA and patients with whole ON (p < 0.05). The alkaline phosphatase activities in patients with alcohol-induced and idiopathic ON also were decreased to 9.03 ± 1.20 and 8.51 ± 1.22, respectively (p < 0.05 and p < 0.05, respectively). For patients with steroid-induced ON, the mean alkaline phosphatase activity in patients with steroid-induced ON was not different compared to patients with OA. When the mean alkaline phosphatase activities from ON patient groups were compared, there was a significant difference between patients with steroid-induced ON and those with alcohol-induced or idiopathic ON (p < 0.05 and p < 0.05, respectively). The difference between alcohol-induced ON and idiopathic ON was not significant (p > 0.05).

Adipogenic differentiation ability of MSCs

The mean optical densities in patients with OA and whole ON were 0.63 ± 0.13 and 0.54 ± 0.21, respectively. The difference in optical density was not statistically significant between patients with hip OA and patients with whole ON (p > 0.05). For patients with alcohol-induced, idiopathic and steroid-induced ON, the mean optical density was 0.47 ± 0.25, 0.56 ± 0.17 and 0.62 ± 0.16, respectively. The difference in adipogenic differentiation (in terms of the optical density) was not statistically significant between patients with alcohol-induced, idiopathic or steroid-induced ON and patients with OA (p > 0.05, p > 0.05 and p > 0.05, respectively). When the mean optical density from the ON patient groups was compared, there was no significant difference (p > 0.05).

CONCLUSION:
Our results indicate that osteogenic differentiation ability in MSCs is related to nontraumatic ON of the femoral head and the differentiation potential of MSCs in patients with nontraumatic ON differs according to its risk factor.

REFERENCES:
2. Jones. 2001 A Textbook of Rheumatology