METABOLIC EFFECTS OF X-RAY IRRADIATION ON ADULT HUMAN ARTICULAR CHONDROCYTES
*Takahashi, Yoshiaki; *Toda, Michio; *Ogawa, Yasuhiro; *Tani, Toshikazu; *Yamamoto, Hiroshi; *Yoshida, Shoji
+Kochi Medical School, Oko-cho, Nankoku, Kochi 783-8505, Japan.

Introduction
Radiation therapy is currently used to treat patients with osteosarcoma, or musculoskeletal diseases and when a soft tissue tumor involves the bone. However, there are risks of causing injury to the articular cartilage by irradiation if the malignant tumor is near an articular joint. However, there have been few reports regarding the metabolic effects of X-ray irradiation on adult human articular chondrocytes. The purpose of this study was to evaluate whether X-ray irradiation during tumor surgery might impair chondrocyte metabolism by measuring apoptosis, and production of chondroitin sulfates and prostaglandin E2 (PGE2) in chondrocytes from normal and degenerated of cartilage after exposure to X-ray irradiation.

Materials and Methods
Isolation of Chondrocytes
Articular cartilage was obtained at the time of surgery from patients undergoing total knee arthroplasty. Full thickness cartilage was harvested, and degenerated and non-degenerated areas (determined by macroscopic findings) and the chondrocytes were isolated at 37 ºC with 5% CO2 overnight. Isolated chondrocytes were washed twice with PBS (Phosphate Buffered Saline), and the aliquots were divided into 8 dishes and cultured in DMEM (Phosphate Buffered Saline), and the aliquots were divided into 8 dishes and cultured in DMEM.

Irradiation of Chondrocytes
Irradiation was administered to the cultured chondrocytes with 4-MV X-rays on a ML-15 MDX linear accelerator (Mitsubishi Electric Co., Ltd, Tokyo, Japan) at doses of 0, 10, 20, and 30 Gy (dose rate: 3.5 Gy/min). The dishes were then returned to the incubator and cultured in a humidified atmosphere of 5% CO2 in air at 37 ºC for 24 or 48 hours.

Annexin V-FITC flow-cytometric analysis
Annexin V was used as an indicator of apoptosis. Annexin V is a Ca-dependent, phospholipid-binding protein with high affinity for phosphatidylserine (PS) that binds to cells with exposed PS. In the early stages of apoptosis, PS is translocated from the inner part of the plasma membrane to the outer layer, becoming exposed at the external surface of the cell. Annexin V can be used as a sensitive probe for PS exposure on the outer leaflet of the cell membrane, an early indicator of apoptosis.

To quantify apoptotic cells, cultured chondrocytes were washed twice with cold PBS then resuspended in a 1X binding buffer (500 µl for each sample). The binding buffer with the cells was passed through a nylon mesh and centrifuged. The cell pellets were then resuspended in 100 µl of 1X binding buffer and 5 µl of Annexin V-FITC and 10 µl of PI were added to each tube. The cells were then gently mixed and incubated for 15 minutes at room temperature in the dark. An additional 400 µl of 1X binding buffer was added to each tube, and within 30 minutes the contents of each tube were analyzed by flow cytometry. To determine the threshold of apoptosis, an Annexin V blocking procedure was performed using recombinant Annexin V. For the control, cells stained with Annexin V-FITC alone (no PI), and those stained with PI alone (no Annexin V-FITC) were examined. The apoptosis ratio was analyzed quantitatively using FACSort (BECTON DICKINSON) with CELLQuest software.

Glucosaminoglycan production
After chondrocytes were cultured for 48 hours, the conditioned medium was removed and stored at −20 °C and digested with chondroitinase ABC, and hyaluronidase derived from Streptococcus dysgalactonii. The concentration of total chondroitin, chondroitin−6 sulfate (C6S) and chondroitin−4 sulfate (C4S) in the culture medium were measured by high-performance liquid chromatography.

PGE2 production
After incubation of the chondrocytes for 48 hours, PGE2 in the culture medium was measured with the PGE2 Enzyme Immunoassay Kit.

Statistical analysis
Mean and S.E.M of biological markers were calculated. A two-way ANOVA (Dunnett method) was applied to determine whether there were significant (p<0.05) differences in the biological measures between degenerated and non-degenerated chondrocytes, and among the different irradiation doses.

Results
There were no differences between non-degenerated and degenerated chondrocytes groups in the number of annexin V-positive cells. The number of cells stained positively for Annexin V did not increase with the irradiation dose in either group. The production of C-6S and C-4S in the non-degenerated chondrocyte group was greater than that in degenerated group. The production of C-6S and C-4S was not altered by irradiation dose in either group. The concentration of PGE2 in non-degenerated chondrocytes increased with 30 Gy radiation (p=0.03), as compared with no radiation (Fig 1). Furthermore, the concentration of PGE2 increased in a dose-dependent manner in degenerated chondrocytes with 20 (p=0.04), and 30 Gy (p=0.037) radiation (Fig 1).

Discussion
Previous studies have demonstrated, using fluorescence immunostaining, that apoptosis was less frequent in non-degenerated chondrocytes than in degenerated chondrocytes with a fluorescent microscope. In this study, FACSort was used for quantitative analyses of apoptosis in irradiated chondrocytes. The present study is contradictory to our previous findings.

The sensitivity of chondrocytes to irradiation changes with the stage of cell differentiation. It has been demonstrated that 10Gy irradiation does not affect the metabolism of proteoglycans or type II collagen in cultured mature articular chondrocytes.

The effect of X-ray irradiation on the function of chondrocytes is still controversial. Previous studies have demonstrated the inhibition of glycosaminoglycan synthesis in a dose- and time-dependent manner. In the present study the synthesis of CS6, which is a mature aggrecan and the main CS isomer in human articular cartilage, was not affected by radiation in culture. C4S, an immature aggrecan detected in serum, synovium and the meniscus, was also not affected by radiation.

There have been few reports regarding PGE2 production in irradiated chondrocytes. Prostaglandins inhibit proteoglycan and protein synthesis and cause a decrease in articular cartilage mass. The present study demonstrated that PGE2 concentration after irradiation in degenerated chondrocyte cultures was greater than that in the non-degenerated chondrocyte group. PGE2 in degenerated chondrocytes increased in a radiation dose-dependent manner suggesting that high doses of X-ray radiation may induce inflammation and inhibit proteoglycan synthesis in degenerated chondrocytes. Therefore, it is important to consider the existence of osteoarthritis of articular joints in the area of radiation when radiation therapy is used during tumor surgery.

Figure 1: The concentration of prostaglandin E2 in cultures after irradiation of non-degenerated chondrocytes, and degenerated chondrocytes was determined. Statistical comparisons between these groups were made using a two-way ANOVA. * p<0.05, ** p<0.01.