Acridine orange induces binucleation in chondrocytes


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Summary

Objective: Although it is well known that binuclear cells commonly appear among the chondrocytes of normal cartilages as well as among neoplastic chondrocytes of chondrosarcomas, the mechanism of binucleation is still unclear. Therefore, this study was undertaken to clarify the mechanism of binucleation in chondrocytes, using primary culture cells of growth plate cartilage.

Design: These chondrocytes were exposed to acridine orange (AO) which is a fluorescent dye for differentiating certain DNAs and RNAs in nuclei and cytoplasm, and which inhibits mitosis. After exposure to 0.5 μg/ml AO, for 0, 6, 24, 48, and 96 h, the following parameters were investigated: (1) cell growth rate (GR); (2) frequency of hyperdiploid cells (%HDC) by DNA cytofluorometry; (3) mitotic index (MI); (4) BrdU labeling index (LI); (5) frequency of binuclear cells (%BNC).

Results: Compared with the control cells, which were cultured in AO-free medium, the GR was remarkably inhibited at 24 h. MI was also decreased from 6 to 24 h, and LI decreased at 48 h. However, these parameters were recovered at 96 h. The %HDC was increased from 6 to 96 h, and the %BNC was also increased to a maximum of six times that of the control cells at 96 h.

Discussion: These results suggested that the binuclear cells observed among the cultured chondrocytes may be formed from G2 arrested cells by amitotic nuclear division, but not by mitosis without cytoplasmic division or cell fusion. © 2001 OsteoArthritis Research Society International

Key words: Chondrocytes, Growth plate, Binuclear cells, Acridine orange.

Introduction

Although many investigators have reported the presence of binuclear cells among the chondrocytes of normal cartilages1–5 as well as among the neoplastic chondrocytes of chondrosarcomas6–11, the mechanism of binucleation is still unclear6. It may be caused by nuclear division without division of the cytoplasm, by cell fusion, or by amitotic nuclear division. We have recently found many binuclear cells among isolated and smeared cells in giant cell tumor of bone (GCT), in vivo as well as in vitro12,13. We presumed that these binuclear cells of GCT might have developed via the mechanism of amitotic nuclear division after G2 arrest, based on the results of in-vitro study with exposure to acridine orange (AO), which is a fluorescent dye for differentiating certain DNAs and RNAs in nuclei and cytoplasm, and which inhibits mitosis14–20. Therefore, we conducted the present study to clarify the mechanism of binucleation in chondrocytes by AO-exposure technique.

Methods

Chondrocytes were isolated from growth plate cartilage of the calf proximal tibia by overnight digestion of the matrix with 1% collagenase (CLS II, Worthington, U.S.A.), minced and cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C and 5% CO2 atmosphere in culture dishes (the 6-well plates). After administration of 0.5 μg/ml AO (Sigma, U.S.A.) to the cultured cells in each plate all cells, including the mitotic cells, were harvested from both the culture medium and plates by trypsinization at 0, 6, 24, 48, and 96 h. Cell kinetic analysis was performed to determine the following parameters: (1) cell growth rate, (2) frequency of hyperdiploid cells by DNA cytofluorometry, (3) mitotic index,
(4) BrdU labeling index, and (5) frequency of the binuclear cells. These studies were repeated five times, and the value of each parameter is expressed as the mean with standard deviation.

Cell growth rate (GR)

The viable chondrocytes isolated from each medium (including the mitotic cells) and plate by trypsinization were counted sequentially with a hemocytometer by the trypan blue exclusion test, and GR was assessed by ratio where the total living cell number of each plate was divided by the mean cell number of 10 plates at the beginning of AO exposure.

Mitotic index (MI)

After measurement of viability the remaining isolated cells were smeared on glass slides using a centrifugal automatic smear machine, followed by drying and fixation with 70% ethanol, which removes AO completely. These smeared cells were stained with DAPI (4′,6-diamidino-2-phenylindole) at 1 μg/ml. Under a fluorescence microscope, the MI (frequency of nuclear mitotic figures per 1000 cells after counting 10,000 cells) was measured in each slide.

BrdU labeling index (LI)

To examine DNA synthetic activity of the chondrocytes, an immunofluorescence method with bromodeoxyuridine
(BrdU) was performed. Before cell harvesting, the cells were exposed to 10 μmol BrdU for 30 min. After preparing cell smears and fixation with buffered formalin, the specimens were treated with 4N HCl followed by washing twice with PBS. The mouse monoclonal Ig G antibody to BrdU (Becton Dickson, U.S.A.) was added to the specimens for 1 h. After washing out the antibody with PBS, the FITC-labeled antibody to the mouse Ig G (Becton Dickson) was added for 1 h. The LI (per 100 cells after counting 10,000 cells) was measured under a fluorescence microscope.

Frequency of hyperdiploid cells (% HDC)

To analyse the sequential changes of DNA ploidy in the cultured chondrocytes, DNA cyt fluorometry was carried out12,21–24. The nuclear DNA content of each cell, stained with DAPI, was measured using an epi-illumination type cyt fluorometer (fluorescence microscope and photomultiplier; Nikon SPM RFI-D or OPTIPHOTO with P1, Nikon, Japan) as the fluorescence intensity of DAPI while observing the cell morphology. The cell smears on the glass slides were excited by UV light (365 nm) and each cell emitted blue fluorescence (400 nm) from DAPI binding to DNA. In each specimen 300 mononuclear chondrocytes were measured and their data automatically entered in a personal computer (9800 VM2, NEC, Japan) to plot a DNA content histogram from which the cell kinetics were assessed. The frequency of hyperdiploid cells having DNA content greater than diploidy (2c) was calculated by our method (5) and expressed as % HDC. We always measured the DNA content of the untreated cells before these studies to determine the ploidy class of 2c.

Frequency of binuclear cells (% BNC)

After continuous exposure to 0.5 μg/ml AO, the cells were smeared on the glass slide by the centrifugal method and stained with hematoxylin-eosin. The morphology of each cell was observed under the microscope and % BNC in all cells (10,000 cells) was calculated after 0, 6, 24, 48, and 96 h.

Results

At 96 h after AO exposure the number of binuclear cells in chondrocytes was increased. Figure 1 shows representative mononuclear and binuclear chondrocytes which were stained with H-E after cell isolation and smear preparation.

Compared with the control cells, which were cultured in AO-free medium, GR of the cultured chondrocytes exposed to AO was inhibited by one half (Fig. 2). However, at 96 h, GR had almost recovered. MI of the control cells was highest at 48 h and gradually decreased later, while MI of the cells exposed to AO was from 6 to 24 h, suggesting that AO inhibited the mitotic activity (Fig. 3). Nevertheless, MI also recovered at 48 h. LI of the chondrocytes exposed to AO was temporarily increased at 6 h, but was decreased at

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Fig. 6. Nuclear DNA histograms of the control cells (A) and AO-exposed cells (B) at 48 h after AO exposure. There are many tetraploid cells arrested at G2 phase of the cell cycle among the AO-exposed chondrocytes.

Fig. 7. Sequential changes in the % BNC (mean±s.d.) of the control cells and AO-exposed cells (N=5 in each group). During 24 to 96 h, there was a significant difference between control and AO-exposed cells (P<0.001).
48 h, indicating that AO inhibited DNA synthetic activity (Fig. 4). This parameter also recovered at 96 h. The % HDC of mononuclear chondrocytes exposed to AO was increased at 6 h and was maintained high until 96 h (Fig. 5). On the DNA histogram there were many arrested cells at G2 phase of the cell cycle, suggesting that AO induced G2 arrest in the cultured mononuclear chondrocytes (Fig. 6). The %BNC of the control cells gradually increased from 0.5 % at 0 h to 1.2% after 96 h, whereas that of the cells exposed to AO was sequentially and remarkably increased from 0.5% at 0 h to 4.5% at 96 h (Fig. 7). These results indicated that AO induced binucleation in the cultured chondrocytes.

Discussion

There have been many reports on the binuclear cells among the chondrocytes of the ear and articular cartilages1–5, as well as among the neoplastic chondrocytes of chondrosarcomas6–11. Especially, the ear cartilage chondrocytes have a single nucleus in the new-born rabbit, but many become binuclear after the age of one month5. The presence of the binuclear cells is an important diagnostic factor for distinguishing chondrosarcomas from chondroma6–11. However, neither the biological significance nor the mechanism of formation of binuclear chondrocytes has been clarified5.

In this study we attempted to clarify the mechanism of binucleation of chondrocytes, using AO. The results showed that AO induced binucleation of the cultured chondrocytes of the calf growth plate cartilage. AO also inhibited DNA synthesis and nuclear mitosis, and consequently induced G2 arrest followed by cell growth inhibition, although these effects were recovered within 96 h even with continuous exposure to AO. In view of the inhibitory effect of AO on mitosis, it seems unlikely that the binuclear chondrocytes are formed by nuclear mitosis without cytoplasmic division. Each nucleus of the binuclear cells was found to be diploid and the actual mechanism of amitotic division is not yet apparent. Such mechanisms might include cleaving or torsion of nuclear membrane to evenly divide. Malignant tumor cells sometimes show irregular lobulation of the nucleus such as micronucleus, suggesting direct nuclear division without mitosis. Cell fusion with mononuclear cells is conceivable25, but AO has not been reported to have such an effect14–21.

Our hypothesis that binucleation of chondrocytes may be caused by amitotic nuclear division is supported by the chromatin bridge between the cleaving nuclei illustrated in Fig. 8.

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References


