Introduction: Chondrosarcoma is second to osteogenic sarcoma in frequency as a malignant tumor of bone. It is difficult to obtain wide surgical resection margins because of its location adjacent to neurovascular and visceral structures. Neither irradiation nor chemotherapy seems to be an effective treatment for chondrosarcoma; thus new protocols are needed. We reported gene therapy of chondrosarcoma using retrovirus vector encoding suicide gene at the 45th annual meeting of ORS. Our model system using gene therapy demonstrated that the strong bystander tumouricidal effect was shown in vitro and tumor size was reduced markedly in vivo. The mechanism of bystander effect is still unclear. Metabolic cooperation mediated by the gap junction is suggested as a major contributor to the phenomenon. However, chondrosarcoma cells contain an extracellular matrix, which may inhibit the transport of substrates from cells to cells. In order to clarify the mechanism of the bystander effect, we established a 3-dimensional cell culture system, simulating chondrosarcoma in vivo. In this study, the bystander effect was investigated in cell culture system and in vivo tumor.

Materials and Methods:
Tumor cells and animals: Human chondrosarcoma cell line HCS-TG was used. Male ICR nu/nu nude mice were used to determine in vivo experiments.

Production of recombinant retrovirus vectors and retrovirus
Infection: We produced recombinant retrovirus vectors (pLTRNL) bearing a herpes simplex virus thymidine kinase (HSV-Tk) gene. The strategy is to infect chondrosarcoma cells by virtue of HSV-TK gene expression, and to sensitize them to antitherapeutic drug, ganciclovir (GCV). Ganciclovir is a guanosine analog, which is metabolized to a cytotoxic product by HSV-Tk. In the other vector (LZRNL) the HSV-Tk gene was replaced by Escherichia coli beta-galactosidase gene (lacZ gene). To produce a transmissible virus, vector DNA was transfected with the calcium phosphate coprecipitation method into amphotrophic producer cells (PA317). The human chondrosarcoma cells (HCS-TG) were plated in 10 cm dishes and infected 24h later by exposure for 48h to virus from the PA317/LTRNL vector-producer line or the PA317/LZRNL in the presence of 8μg/ml polybrene. These were cultured with previous medium containing the G418 (neomycin analogue) The G418-resistant clones of HCS-TG/LTRNL (HCS-TG-tk) and HCS-TG/LZRNL (HCS-TG-Z) were selected randomly from the surviving colonies and used in the following experiments.

Collagen Gel Embedded Culture method: 3-dimensional cell culture using collagen gel was applied in this study. Three layers were made in 3.5 cm dishes. Top layer was made of HCS-TG cells embedded with collagen gel (from pig type I collagen), RPMI 1640 and HEPES in 3.5 cm dishes. And in another experiments of bystander effect cells at the concentration of 10^5 cells per 35-mm well. Further, tk+ cells were cocultured with tk- cells (lacZ+) at ratios of 1:0, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, 1:20, for a total of 3 experiments.

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GV sensitivity and bystander tumoricidal effect: The cytotoxicity of the nucleoside analog ganciclovir (GCV) was determined by using a tetrazolium-based colorimetric assay (MTT assay). We examined in vitro bystander tumoricidal effect by coculturing with HSV-TG-Z and HCS-TG-G4k. For cell survival analysis in coculture, cells were quantitated directly on culture plates after staining for X-gal in vivo.

Tumor cell lines HCS-TG-tk and HCS-TG-Z were cocultured respectively at a moderate density in medium containing 10 μM GCV for 5 days. Further, tk+ cells were cocultured with tk- cells (lacZ+) at ratios of 1:0, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, 1:20, for a total of 3 x 10^6 cells per 35-mm well. And in another experiments of bystander effect cells were incubated with TNF at final concentration of 1000 ng/ml.

In vivo experiments: Chondrosarcoma (HCS-TG) was implanted in 20 nude mice (male ICR, nu/nu). When the tumors grew to approximately 250 mm^3 (60x70mm), the animals were divided into two groups. Ten tumors were injected with 1x10^5 HSV-TG-G4k cells subcutaneously. An additional 10 tumors were injected in a similar manner with HCS-TG-Z cells subcutaneously. One week after injection, nude mice were injected twice daily intraperitoneally for 14 days with GCV at 150 mg/kg of body weight. Tumor size was measured with calipers twice a week from day 0 (first ganciclovir treatment) to day 28. On day 14, some tumor cells injected with HCS-TG-tk and HCS-TG-Z were resected and stained with H.E. and X-gal.

Results:
GV sensitivity and bystander tumoricidal effect: The cytotoxic activity of GCV was dose-dependent in the HSV-tk gene-transduced clones of the human chondrosarcoma cell line. However, no effect was shown in cells without gene transduction. 97% of the cells were killed at the concentration of 1 μM of GCV, and 99% of the cells were killing at 10 μM. The cytotoxic activity of GCV was observed in 3-dimensional cell culture. We observed the bystander effect at various cell ratios in cocultures of HCS-TG-tk and HCS-TG-Z. At a ratio of HCS-TG-tk: HCS-TG-Z cells of 1:0 to 1:10, 80% of cells were killed (Fig 1). This experiment showed strong bystander effect in 3-dimensional cell culture. And this phenomenon was boosted by TNF.

Conclusion and discussion: Understanding the mechanism of the bystander effect is very important of cancer gene therapy. It has been emphasized that gap junctional communication plays an important role in the bystander effect. In this study, however, the bystander effect was observed even in 3-dimensional cell culture without gap junction. The gap junction was not always necessary to the bystander effect. So it has shown that another mechanism such as apoptosis, endocytosis of toxin cell debris, soluble toxins (TNF) have been worked.