学 位 論 文 の 要 旨

氏名　　稲尾　瞳子

学　位　論　文　名　Antitumor Effects of Cytoplasmic Delivery of an Innate Adjuvant Receptor Ligand, Poly(I:C), on Human Breast Cancer

発　表　雑　誌　名　Breast Cancer Research and Treatment
（巻，初頁～終頁，年）　(Published online: 28 December 2011)

著　　者　　名　Touko Inao, Nanae Harashima, Hiroyuki Monma, Shinji Okano, Masayuki Itakura, Tsuneo Tanaka, Yoshitsugu Tajima, Mamoru Harada

論 文 内 容 の 要 旨

INTRODUCTION

Innate adjuvant receptors, including toll-like receptors (TLRs), play a crucial role in many aspects of immune response. These receptors are broadly expressed in immune cells and the initiation of adjuvant receptor signaling induces the release of inflammatory cytokines, the maturation of dendritic cells, and the activation of adaptive immunity. In addition to immune cells, various types of cancer cells have been reported to express functional innate adjuvant receptors, and the majority of these studies has been focused on TLRs. Among a panel of TLRs, TLR3 has been suggested to be therapeutically useful. Polyinosinic-polycytidylic acid [poly(I:C)], which is a ligand for endosomal adjuvant receptor TLR3, can induce the expression of inflammatory cytokines and type I interferon, thus enhancing the antitumor immune responses. Alternatively, poly(I:C) can be a ligand for melanoma differentiation-associated gene (MDA) 5, which is another cytoplasmic adjuvant receptor. Interestingly, a previous study has shown that poly(I:C) transfection is accompanied by autophagy, which functions cytoprotectively under starvation and stress conditions. However, the precise roles of autophagy in treatment-associated cancer cell death have not yet been fully elucidated. In the present study, we explored whether delivery of poly(I:C) into the cytoplasm of three human breast cancer cell lines could induce

antitumor effects and attempted to elucidate the roles of autophagy in cancer cell death after poly(I:C) transfection.

MATERIALS AND METHODS

Three breast cancer cell lines (MCF-7, MDA-MB-231, and BT-549) were used in this study. Transfection of poly(I:C) was performed using X-tremeGENE transfection reagent. Cell viability was measured using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) kit. Apoptosis was measured using the Annexin V-FITC Apoptosis Detection Kit and propidium iodide (PI). Cell proliferation was estimated by a FACSCalibur flow cytometer using the Ki-67 staining kit and the CellTrace^{TM} carboxyfluorescein diacetate, succinimidyl ester (CFSE) cell proliferation kit. The following primary antibodies were used for immunoblot: anti-TLR3, anti-MDA5, anti-caspase-3, anti-caspase-7, anti-caspase-8, anti-caspase-9, anti-LC3, anti-cyclinD1, anti-c-Myc, anti-p21$^{Waf1/Cip1}$, anti-p27^{kip1}, anti-beclin-1, anti-β-actin, and anti-α-tubulin. Goat anti-rabbit or goat anti-mouse alkaline phosphatase-conjugated secondary antibodies were used to detect the primary antibodies. Transfection of siRNA was performed using Lipofectamine^{TM} RNAiMAX. To detect autopahgy, cancer cells were transfected with the pcDNA3.1/NT-GFP-TOPO vector encoding LC3B (NM_022818) using Lipofectamine 2000. After incubation with Hoechst 33342, confocal imaging was observed using an Olympus FV1000-D laser scanning microscope. In an *in vivo* xenograft model, BALB *nu/nu* female mice were inoculated with MDA-MB-231 (5×10^6 cells) into the right mammary fat pad. Thereafter, *in vivo* transfection of poly(I:C) was performed using *in vivo*-jetPEI^{TM} transfection reagent four times, and the tumor size was measured twice weekly. This *in vivo* experiment was performed according to the ethical guidelines for animal experiments of the Shimane University Faculty of Medicine (approval number: IZ23-70).

RESULTS AND DISCUSSION

Three breast cancer cell lines expressed TLR3 and MDA5. Selective knockdown of MDA5, but not TLR3, partially but significantly restored the viability of all breast cancer cell lines after poly(I:C) transfection, indicating that the MDA5 was at least partially responsible for the

antitumor effects following poly(I:C) transfection. Analysis on apoptosis revealed that poly(I:C) transfection significantly increased the percentages of PI^- Annexin V^+ (early apoptosis) and PI^+ Annexin V^+ (late apoptosis) cells of all cell lines. In immunoblot assay, apparent cleavages of caspase-3 and caspase-9 were detected in poly(I:C)-transfected MDA-MB-231 and BT-549 cells, whereas cleaved caspase-7 was detected in poly(I:C)-transfected MCF-7 cells. We also evaluated whether poly(I:C) transfection affected the proliferative capacity of surviving cancer cells and found that the expression level of Ki-67, a proliferation-related nuclear protein, in all cell lines decreased after poly(I:C) transfection, and that poly(I:C) transfection decreased the proliferative capacity of CFSE-labeled breast cancer cells. Additionally, in immunoblot assay, poly(I:C) transfection resulted in decreased expression of c-Myc in all cell lines and decreased expression of cyclinD1 in MCF-7 and MDA-MB-231 cells. We also explored the possibility that autophagy played a role in our experimental system. The results were that, although autophagy in MCF-7 was induced after poly(I:C) transfection, the constitutive expression of autophagy was observed in both MDA-MB-231 and BT-549 cell lines. We also examined the effect of knocking down beclin-1, which is essential for autophagy, on apoptosis of breast cancer cells after poly(I:C) transfection and found that the selective knockdown of beclin-1 significantly increased the percentage of poly(I:C) transfection-induced apoptosis in all breast cancer cell lines. In a xenograft mouse mode, the growth of MDA-MB-231 cells was significantly inhibited by *in vivo* poly(I:C) transfection.

CONCLUSION

These findings indicate that cytoplasmic delivery of poly(I:C) can induce apoptosis and growth arrest of human breast cancer cells, and that therapy-associated autophagy prevents apoptosis. The results of this study suggest that innate adjuvant receptors are promising targets and that their ligands could serve as antitumor reagents, which have the potential to simultaneously induce antitumor effects and activate immune cells.