Roles of the PI3K/Akt pathway and autophagy in TLR3 signaling-induced apoptosis and growth arrest of human prostate cancer cells

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Running title: Antitumor effect of TLR3 signaling on prostate cancer

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Abstract

Toll-like receptors (TLRs) are widely expressed in immune cells and play a crucial role in many aspects of the immune response. Although some types of TLRs are also expressed in cancer cells, the effects and mechanisms of TLR signaling in cancer cells have not yet been fully elucidated. In the present study, we analyzed the effects of polyinosinic-polycytidylic acid [poly(I:C)], a TLR3 ligand, on three TLR3-expressing human prostate cancer cell lines (LNCaP, PC3, and DU145). We then further characterized the underlying mechanisms, focusing on the poly(I:C)-sensitive LNCaP cell line. Poly(I:C) significantly reduced the viability of LNCaP cells TLR3- and endosome-dependently. One mechanism for the antitumor effect was caspase-dependent apoptosis, and another mechanism was poly(I:C)-induced growth arrest. Cell survival and proliferation of LNCaP cells depended on the PI3K/Akt pathway and PI3K/Akt inhibitors induced apoptosis and growth arrest similarly to poly(I:C) treatment. Additionally, poly(I:C) treatment caused dephosphorylation of Akt in LNCaP cells, but transduction of the constitutively active form of Akt rendered LNCaP cells resistant to poly(I:C). Immunoblot analysis of proliferation- and apoptosis-related molecules in poly(I:C)-treated LNCaP cells revealed participation of cyclinD1, c-Myc, p53, and NOXA. Interestingly, poly(I:C) treatment of LNCaP cells was accompanied by autophagy, which was cytoprotective toward poly(I:C)-induced apoptosis. Together, these findings indicate that TLR3 signaling triggers apoptosis and growth arrest of LNCaP cells partially through inactivation of the PI3K/Akt pathway, and that treatment-associated autophagy plays a cytoprotective role.

Key words: TLR3, Prostate cancer, Apoptosis, Autophagy, Akt

Introduction

Toll-like receptors (TLRs) play a crucial role in many aspects of the immune response [1]. TLRs are broadly expressed in immune cells, particularly in antigen-presenting cells, and recognize pathogen-associated molecular patterns such as LPS, viral double-stranded RNA, and unmethylated CpG islands. Initiation of TLR signaling induces release of inflammatory cytokines, maturation of dendritic cells (DC), and activation of adaptive immunity.

In addition to immune cell types, various types of cancer cells have been reported to express functional TLRs. LPS, a TLR4 ligand, activates TLR4-expressing human head and neck squamous cell carcinoma and ovarian cancer cells to promote tumor development and protect against immune attack and chemotherapy [2, 3]. TLR4 signaling promotes escape of human lung cancer cells from the immune system by inducing immunosuppressive cytokines and promoting resistance to apoptosis [4]. In contrast, TLR3 signaling induces antitumor effects. TLR3 may be a potential therapeutic target in clear-cell renal cell carcinoma and melanoma [5, 6] and TLR3 signaling triggers apoptosis in human breast cancer cells [7]. Additionally, TLR3 signaling has been reported to induce apoptosis of human prostate cancer cells through protein kinase Cα (PKCα) activation [8]. Furthermore, it has been reported that TLR3 expression on human breast cancer tissues can be a biomarker for the therapeutic efficacy of adjuvant treatment with double-stranded RNA [9]. These lines of evidence suggest that TLR3 could be a promising therapeutic target molecule for treatment of cancer.

Polyinosinic-polycytidylic acid [poly(I:C)], a TLR3 ligand, can induce expression of inflammatory cytokines and type I IFN through the NF-κB, MAPK, and IFN regulatory factor 3 pathways [10], enhancing the antitumor immune response. Additionally, poly(I:C)-induced type I IFN can augment antitumor immune surveillance while mitigating

regulatory T cells in tumor-bearing hosts [11]. Considering the direct antitumor effects of TLR3 signaling, poly(I:C) is a promising agent for use in simultaneous activation of the immune system and initiation of cancer cell death. In the present study, we investigated the effects of poly(I:C) on three TLR3-expressing human prostate cancer cell lines, and elucidated two underlying mechanisms that regulate the sensitivity of LNCaP cells to poly(I:C)-dependent apoptosis and growth arrest. The antitumor effects of poly(I:C) on LNCaP cells were exerted partially through inactivation of the PI3K/Akt pathway, which is an essential survival mechanism in many human cancers [12, 13]. Additionally, caspase-dependent apoptosis of poly(I:C)-treated LNCaP cells was accompanied by autophagy, which played a protective role in poly(I:C)-induced apoptosis.

Materials and Methods

Cell lines

Three human prostate cancer cell lines, LNCaP, PC3, and DU145, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Invitrogen, Grand Island, NY) and 20 μg/ml gentamicin (Sigma-Aldrich) at 37°C in a humidified atmosphere with 5% CO_2. LNCaP cells expressing a mouse ecotropic receptor were established by transfection with a pcDNA3-based vector containing mouse cationic amino acid transporter-1 [14]. Thereafter, transfected cells were further transduced with a pWZLneo-myr-Akt1 retroviral vector (Cell Biolabs, Inc., San Diego, CA) to establish LNCaP cells expressing a constitutively active form of Akt, designated myr-Akt-LNCaP cells. To improve transduction efficiency, PLAT-E cells [15] were used as packaging cells.

Cell viability assay

Cell viability was measured using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) assay (Nacalai Tesque, Kyoto, Japan). Briefly, cells were seeded in flat-bottomed 96-well plates. On the following day, poly(I:C) (InvivoGen, San Diego, CA) was added to the wells, and then cells were cultured for an additional 2 days. Cells were subsequently incubated in WST-8 and plates were read at a wavelength of 450 nm after 3 h. For inhibition assays, the following reagents were added at the time of poly(I:C) addition: bafilomycin A1 (Sigma-Aldrich), chloroquine (Jena Bioscience, Jena, Germany), z-VAD-fmk (R&D Systems, Minneapolis, MN), rapamycin (LC Laboratories, Woburn, MA), 3-methyladenine (3-MA) (Sigma-Aldrich), LY294002 (SA Biosciences, Frederick, MD), and AKT1/2i (Calbiochem, Darmstadt, Germany).

Semiquantitative RT–PCR analysis

Total RNA was extracted using the PureLink RNA Mini Kit (Invitrogen) and was reverse transcribed using the SuperScript VILO cDNA synthesis kit (Invitrogen) according to the manufacturer's protocols. PCR amplification was performed using Platinum *Taq* DNA polymerase (Invitrogen). The primers used for RT–PCR were as follows: TLR3, 5′-ACAACTTAGCACGGCTCTGGA-3′ (forward) and 5′-ACCTCAACTGGGATCTCGTCA -3′ (reverse); GAPDH, 5'-ACAACAGCCTCAGATCATCAG-3′ (forward) and 5′-GGTCCACCACTGACACGTTG-3′ (reverse).

Flow cytometry analysis

Apoptosis was measured using the Annexin V-FITC Apoptosis Detection Kit (BioVision, Mountain View, CA) and propidium iodide (PI). Analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, Fullerton, CA). Expression of Ki-67 was examined using a Ki-67 staining kit (BD Biosciences, San Jose, CA).

Immunoblotting

Cells were lysed with the M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL) containing a protease inhibitor cocktail (Nacalai Tesque). For detection of phosphorylated molecules, a phosphatase inhibitor cocktail (Nacalai Tesque) was also added. Equal amounts of protein were resolved on 4–12% gradient or 12% SDS-PAGE gels, followed by transfer to polyvinylidene fluoride membranes. After blocking membranes, blots were incubated with the indicated primary antibodies: anti-TLR3 (AnaSpec, San Jose, CA), anti-melanoma differentiation associated gene 5 (MDA5; ProSci Inc., Poway, CA), anti-caspase-3 (Cell Signaling Technology [CST], Danvers, MA), anti-polyADP-ribose polymerase (PARP; Travigen, Gaithersburg, MD), anti-caspase-8 (MBL, Nagoya, Japan), anti-caspase-9 (MBL), anti-LC3 (MBL), anti-Akt1/2/3 (Santa Cruz Biotechnology [SCB], Santa Cruz, CA), anti-pAkt (Thr308; CST), anti-pAkt (Ser473; CST), anti-cyclinD1 (CST), anti-c-Myc (Epitomics, Burlingame, CA), anti-$p21^{Waf1/Cip1}$ (CST), anti-$p27^{kip1}$ (CST), anti-β-catenin (CST), anti-p53 (SCB), anti-NOXA (SCB), anti-PUMA (CST), anti-XIAP (CST), anti-Bcl-xL (CST), anti-Mcl-1 (SCB), anti-β-actin (BioLegend, San Diego, CA), and anti-α-tubulin (SCB). Goat anti-rabbit or goat anti-mouse alkaline phosphatase-conjugated secondary antibodies (Invitrogen) were used to detect the primary antibodies.

Transfection of small interfering RNA (siRNA)

Transfection of siRNA was performed using the Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's instructions. Both TLR3 siRNA (sc-36685) and MDA5 siRNA (sc-61010) were purchased from SCB. Both c-Myc siRNA (#6341) and control siRNA (#6568) were purchased from CST.

Confocal imaging for autophagy

LC3B (NM_022818) was amplified by PCR and inserted into the pcDNA3.1/NT-GFP-TOPO vector (Invitrogen) in-frame with the GFP sequence.

Electroporation of LNCaP cells with these plasmids was performed using the Amaxa Device and Amaxa^{R} Cell Line Nucleofector^{R} Kit R (Lonza, Cologne AG, Cologne, Germany). Cells were cultured on round microscope cover glasses in 24 well-plates with the indicated reagents for 2 days. After incubation with Hoechst 33342 (5 μg/ml) for 30 min, cells were fixed with 3% formalin and put on slide glasses with 4 μl mounting medium for fluorescence (Vectashield, Vector Laboratories, Inc., Burlingame, CA). Confocal imaging was obtained using an Olympus FV1000-D by laser scanning microscope (Olympus, Tokyo, Japan).

Statistics

Data were statistically evaluated using unpaired two-tailed Student's *t*-tests. A *p*-value less than 0.05 was considered to be statistically significant.

Results

TLR3-mediated and endosome-dependent antitumor effects of poly(I:C) on LNCaP cells

To assess the effects of TLR3 signaling on human prostate cancer cells, we used three well characterized human prostate cancer cell lines, two of which (LNCaP and PC3) have already been reported to express TLR3 [8]. As shown in Fig. 1a, TLR3 mRNA was expressed in all three cell lines, although relatively weakly in PC3 cells. However, its expression was clearly enhanced by adding poly(I:C). We next assessed the effect of poly(I:C) on the viability of these cell lines. The viability of LNCaP cells, but not of PC3 or DU145 cells, significantly decreased in the presence of poly(I:C) in a dose-dependent manner (Fig. 1b). MDA5, which residues in the cytoplasm, is another receptor for poly(I:C) [16]. LNCaP cells expressed both TLR3 and MDA5, and their protein expression was

selectively knocked down by transfection with TLR3 siRNA and MDA5 siRNA, respectively (Fig. 1c). Knockdown of TLR3 significantly restored the viability of poly(I:C)-treated LNCaP cells. In immune cells, several TLRs, including TLR3, are expressed in the endosome, an acidic subcellular compartment, and acidification is required for TLR activation [17]. The antitumor effect of poly(I:C) on LNCaP cells was mitigated by adding either bafilomycin or chloroquine, inhibitors of endosomal acidification [17] (Fig. 1d).

Caspase-dependent apoptosis in poly(I:C)-treated LNCaP cells

We next tested whether the decrease in viability of poly(I:C)-treated LNCaP cells was due to apoptosis. Poly(I:C) treatment significantly increased the percentage of Annexin V^+ LNCaP cells (Fig. 2a); the percentage of Annexin V^+ PI^+ PC3 cells slightly increased. Cleaved caspase-3 and PARP were clearly detected in poly(I:C)-treated LNCaP cells (Fig. 2b). Additionally, poly(I:C) treatment led to the remarkable induction of caspase-9 cleavage, whereas it induced only a slight increase in both uncleaved and cleaved caspase-8, in LNCaP cells. Adding z-VAD, a pan-caspase inhibitor, significantly reduced the percentage of Annexin V^+ PI^- LNCaP cells after poly(I:C) treatment (Fig. 2c). As poly(I:C) treatment of LNCaP cells may induce apoptosis through Fas/Fas ligand interactions [18], we tested this possibility. Although LNCaP cells expressed both Fas and Fas ligand, the addition of a blocking anti-Fas ligand antibody did not effect the apoptosis of poly(I:C)-treated LNCaP cells (data not shown).

Participation of the PI3K/Akt pathway in the poly(I:C)-induced antitumor effect on LNCaP cells

Although TLR3 has been shown to activate three signaling pathways in immune cells

(i.e., NF-κB, MAPK, and IFN regulatory factor 3) [10], the signaling pathway(s) activated by TLR3 in cancer cells have not been fully elucidated. However, the PI3K/Akt pathway is activated in LNCaP cells due to a deficiency in PTEN (phosphatase and tensin homolog) [19], and Akt influences n cell viability by its activation of proteins that regulate the cell cycle and apoptosis [12, 13]. As shown in Fig. 3a, PI3K and Akt inhibitors promoted apoptosis and decreased Ki-67 expression in LNCaP cells, similar to the effects of poly(I:C) treatment (Fig. 3b). As TLR3 signaling has been reported to induce apoptosis in LNCaP cells via PKCα activation [8], we also examined the effect a PKCα inhibitor as well as a panel of other inhibitors that target molecules in the MAPK and NF-κB pathways. None of the inhibitors impacted the viability of poly(I:C)-treated LNCaP cells (Suppl. Fig. 1). We further explored the effect of poly(I:C) treatment on phosphorylation of Akt, and found that, although Akt (Ser473 and Thr308) was phosphorylated constitutively, phosphorylation was inhibited in a biphasic manner, 2 h and 24 h later (Fig. 3c). As no morphological changes were observed 2 h after the addition of poly(I:C), the second dephosphorylation appeared to be responsible for the poly(I:C)-induced antitumor effect on LNCaP cells. To obtain direct evidence that dephosphorylation of Akt was involved in the poly(I:C)-induced antitumor effect, we established myr-Akt-LNCaP cells in which Akt was constitutively activated by the transduction of myristoylated Akt [20]. As shown in Fig. 3d, phosphorylation of Akt in myr-Akt-LNCaP cells was higher than that in parental and control mock-LNCaP cells, and poly(I:C) treatment for 24 h had no effect on their phosphorylation. Phosphorylation of Akt (Ser473) was slightly augmented in mock-LNCaP cells compared to the parental cells, which was likely due to an artificial effect of retroviral infection. Importantly, myr-Akt-LNCaP cells were significantly more resistant to poly(I:C) than were parental and mock-LNCaP cells. We also tested the effect of caspase inhibition on phosphorylation of Akt in poly(I:C)-treated LNCaP cells, but the addition of z-VAD had no apparent effect on

Akt dephosphorylation (Suppl. Fig. 2a).

Changes in levels of proliferation- or apoptosis-related proteins in poly(I:C)-treated LNCaP cells

Akt plays an important role in cell proliferation in many types of malignancies. Akt promotes expression of cyclinD1 and c-Myc through the PI3K/Akt-mammalian target of rapamycin (mTOR) pathway [12, 13] and suppresses expression of p21Waf1/Cip1 and p27Kip1 [21, 22]. Akt also regulates translocation of β-catenin into the nucleus via suppression of GSK-3β phosphorylation [23]. As shown in Fig. 4a, poly(I:C) treatment caused a decrease in protein expression of both cyclinD1 and c-Myc and an increase in expression of p21Waf1/Cip1 and p27Kip1. The level of nuclear β-catenin increased after poly(I:C) treatment. As shown in Fig. 4b, poly(I:C) as well as PI3K and Akt inhibitors inhibited c-Myc expression, whereas knockdown of c-Myc had no effect on phosphorylation of Akt in LNCaP cells. Additionally, the addition of z-VAD had no effect on a decrease in protein expression of cyclinD1 or c-Myc in poly(I:C)-treated LNCaP cells (Suppl. Fig. 2b).

Akt is also known to play an important role in cell death by suppressing pro-apoptotic molecules, including caspase-9, p53, NOXA, and PUMA [24, 25], and activating anti-apoptotic molecules, such as XIAP, Bcl-xL, and Mcl-1 [26]. Caspase-9 was activated in poly(I:C)-treated LNCaP cells (Fig. 2b). We examined the expression of a panel of pro-apoptotic and anti-apoptotic molecules in the presence and absence of poly(I:C) (Fig. 4c). Poly(I:C) treatment increased the protein expression of p53 and NOXA, but simultaneously increased expression of the anti-apoptotic molecule XIAP. No significant change was observed in the expression of the other anti-apoptotic molecules, Bcl-xL and Mcl-1. We also tested Bad-mediated apoptosis, as Bad is known to participate in

PI3K/Akt-mediated apoptosis [27]. Dephosphorylation of Bad (Ser112 and Ser136) was not observed in poly(I:C)-treated LNCaP cells (data not shown).

Autophagy in poly(I:C)-treated LNCaP cells

Recently, autophagy has received much attention in various fields of cell biology [28]. Thus, we tested whether autophagy was involved in our system. LC3 exists in two forms, LC3-type I, which is cytosolic, and its proteolytic derivative LC3-type II, which localizes to the autophagosomal membrane [29]. Similar to rapamycin, a well-known inducer of autophagy, poly(I:C) treatment increased expression of not only LC3-type I but also LC3-type II (Fig. 5a). The induction of autophagy can also be assessed by confocal imaging of LC3 foci in GFP-LC3 fusion protein-expressing cells. We transiently transfected a plasmid encoding GFP-LC3 into LNCaP cells, and examined expression of GFP-LC3 foci. After treatment with rapamycin, GFP-LC3 foci were detected in GFP-LC3-transfected LNCaP cells but not in GFP/NT-transfected cells (Fig. 5b). Importantly, these GFP-LC3 foci were detected in the cytoplasm of GFP-LC3-transfected LNCaP cells after treatment with a suboptimal dose (5 μg/ml) of poly(I:C), and the addition of 3-MA, an inhibitor of autophagy [30], decreased them (Fig. 5, c and d). When GFP-LC3-transfected LNCaP cells were treated with a higher dose (20 μg/ml) of poly(I:C), no statistical difference in the percentage of GFP-LC3 foci-positive cells was observed between poly(I:C)-treated and untreated LNCaP cells (data not shown), likely due to increased apoptosis of poly(I:C)-treated LNCaP cells. Interestingly, the addition of z-VAD showed a non-significant tendency to increase the percentage of GFP-LC3 foci-positive LNCaP cells after poly(I:C) treatment and significantly increased the number of GFP-LC3 foci (>5) among 20 GFP-LC3 foci positive LNCaP cells (Fig. 5d). Conversely, adding 3-MA significantly increased the percentage of PI^- Annexin V^+ LNCaP cells after poly(I:C) treatment, and this increase was significantly

suppressed by adding z-VAD (Fig. 5e).

Discussion

Human prostate cancer cells express several kinds of TLRs. It has been reported that approximately 80% of prostate carcinomas are positive for TLR3, TLR4, and TLR9 based on an analysis of tumors from 133 patients with prostate cancer [31]. In addition, TLRs on human prostate cancer cells have been reported to be functional. Consistent with our results, TLR3 stimulation with poly(I:C) can induce apoptosis of LNCaP cells [8]. In addition, TLR3 signaling can trigger NF-κB-dependent upregulation of inflammatory molecules and the recruitment of leukocytes, resulting in the stimulation of anti-cancer immune responses [32]. Although there is no report examining the effect of TLR4 signaling on prostate cancer, TLR9 signaling has been reported to stimulate prostate cancer invasion [33]. Thus far, only TLR3 ligand has been demonstrated to induce apoptosis in some human prostate cancer cell lines and to stimulate anticancer immune responses.

Signaling through the PI3K/Akt pathway controls proliferation and apoptosis of cancer cells [12, 13]. Akt mediates these effects by modulating cell cycle and apoptosis regulatory proteins [34], and changes in the corresponding genes have been linked to prostate cancer [35]. Importantly, immunohistochemistry analyses revealed that advanced human prostate cancer is accompanied by the expression of phosphorylated Akt [36], and phosphorylation of Akt (Ser473) is a predictor of poor clinical outcome in prostate cancer [37, 38]. In this study, we showed that constitutive phosphorylation of Akt in LNCaP cells was decreased by poly(I:C) (Fig. 3c). We also examined the susceptibility of myr-Akt-LNCaP cells, in which Akt was constitutively activated, and found that these cells were significantly more resistant to poly(I:C) than parental and mock-LNCaP cells (Fig. 3d). Based on these findings, we

concluded that dephosphorylation of Akt was, at least partially, responsible for the poly(I:C)-induced antitumor effect on LNCaP cells. To the best of our knowledge, this is the first report showing that TLR signaling triggers antitumor effects in human cancer cells through inactivation of the Akt pathway. However, the mechanism by which TLR3 signaling influences the PI3K/Akt pathway in LNCaP cells remains unknown. In HEK293 cells stably expressing TLR3, double-stranded RNA-activated phosphorylation of two specific tyrosine residues in TLR3 was reported to recruit and activate PI3K and its downstream kinase, Akt [39], suggesting an essential role for the PI3K/Akt pathway in TLR3-mediated gene induction. Taking this information into consideration, one can speculate that PI3K may transition from docking with growth factor-dependent receptor tyrosine kinases, which activate the PI3K/Akt pathway in cancer cells, to binding TLR3 as an adaptor molecule after poly(I:C) stimulation. However, further studies are required to elucidate the precise mechanism.

Akt suppresses apoptosis by inhibiting caspase activity [25] and inactivating p53 [24]. In the present study, we showed that poly(I:C) can induce caspase-dependent apoptosis of LNCaP cells (Fig. 2) while increasing the expression of p53 and its target NOXA (Fig. 4c). Expression of p53 in LNCaP cells may account for the difference in susceptibility of the three prostate cancer cell lines. LNCaP cells express wild-type p53 [40] and increased its expression after poly(I:C) treatment (Fig. 4c). Conversely, PC3 cells do not express p53, and a mutated form of p53 has been detected in DU145 cells [40]. We also tested the possibility that Bad participated in poly(I:C)-induced apoptosis, as Bad induces apoptosis by inhibiting the PI3K/Akt pathway in PTEN-deficient human breast cancer cells [27]. However, Bad did not appear to participate in poly(I:C)-induced apoptosis in LNCaP cells.

Akt is also known to promote cell proliferation through activation or suppression of proliferation-related molecules. The kinase mTOR is a downstream target of the PI3K/Akt

pathway that increases expression of cyclinD1 and c-Myc [41]. Additionally, both p21Wsf1/Cip1 and p27Kip1 serve as negative regulators of the cell cycle, and Akt suppresses their function [21, 22]. We showed that expression of cyclinD1 and c-Myc was decreased and that of p21Wsf1/Cip1 and p27Kip1 was increased in poly(I:C)-treated LNCaP cells (Fig. 4a). Since the effects of decreased c-Myc expression was most apparent in cell cycle-related molecules, we also tested the possibility that decreased expression of c-Myc affected the phosphorylation level of Akt in poly(I:C)-treated LNCaP cells, but the results were negative (Fig. 4b). Overall, poly(I:C) treatment shifted the expression pattern of cell death-related and proliferation-related molecules consistent with apoptosis and growth arrest.

Autophagy has been receiving much attention in various fields of cell biology [28]. A primary role of autophagy is its cytoprotective effects under starvation and stress conditions [42]. Therefore, we explored the possibility that autophagy participated in the experimental system, and found that autophagy was induced in poly(I:C)-treated LNCaP cells (Fig. 5). Additionally, poly(I:C)-induced autophagy of LNCaP cells was sustained and/or augmented when apoptosis was inhibited (Fig. 5d). Conversely, the poly(I:C)-induced apoptosis of LNCaP cells was promoted when autophagy was inhibited by 3-MA (Fig. 5e). These findings imply that apoptosis and autophagy are inversely related. Formation of GFP-LC3 foci was observed in GFP-LC3-transfected LNCaP cells when a suboptimal dose of poly(I:C) was added, and the addition of a higher dose of poly(I:C) hindered detection of GFP-LC3 foci due to increased apoptosis. This observation suggests that autophagy may initially be cytoprotective, but when the degree of poly(I:C)-induced stress becomes excessive (*e.g.*, at higher doses of poly(I:C)), autophagy can no longer maintain cell viability, resulting in apoptosis.

Poly(I:C) is a useful immunomodulator that effectively activates professional

antigen-presenting cells, such as DC [10]. Additionally, Akt activation can render cancer cells resistant to antitumor cellular immunity [43], implying that Akt inactivation may increase the susceptibility of cancer cells to immune surveillance. Furthermore, transfection of poly(I:C) into human melanoma cells may induce cell death through the cytosolic poly(I:C) receptor, MDA5 [44, 45]. Together, these lines of evidence and our findings suggest that poly(I:C) is a promising agent that can act not only as an adjuvant to stimulate innate and adaptive immunity, but also as a drug with the potential to induce cell death and growth arrest in cancer cells.

Acknowledgements

We thank Ms. Yasuko Moriyama for her technical assistance. We thank Dr. Davey RA for the mCAT-1 vector and Dr. Toshio Kitamura for the PLAT-E cells. This study was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (no. 18591449 to M. H., no. 23701074 to N. H.), and Extramural Collaborative Research Grant of Cancer Research Institute, Kanazawa University.

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Figure legends

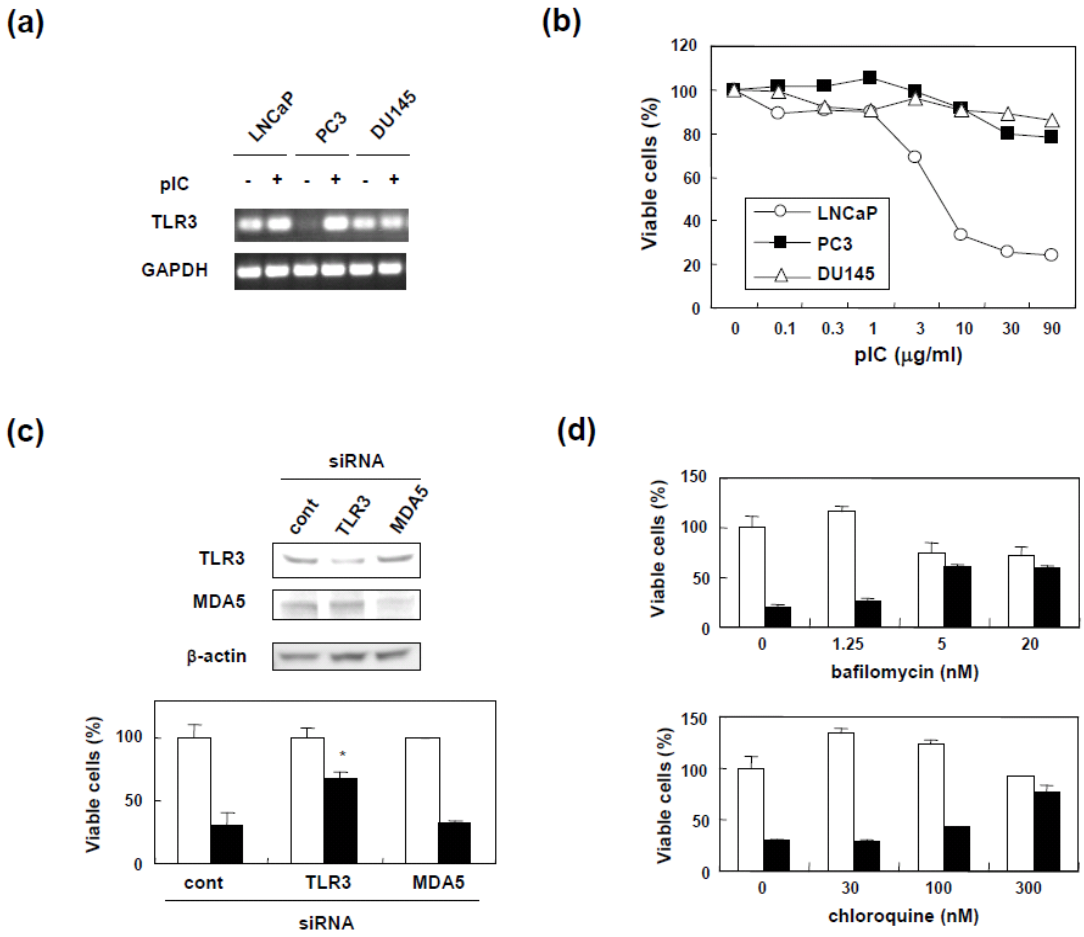

Fig. 1 TLR3-mediated antitumor effect of poly(I:C) on LNCaP cells. **a** RT-PCR was used to assess the expression of TLR-3 in three cell lines in the presence or absence of 10 μg/ml poly(I:C) for 48 h. pIC, poly(I:C). Cell viability (%) was determined using the WST-8 assay. **b** Three cell lines were cultured with the indicated doses of poly(I:C) for 48 h. **c** LNCaP cells were transfected with TLR3, MDA5, or control siRNA, and immunoblotting was performed. Cells were cultured with (black symbols) or without (open symbols) 5 μg/ml poly(I:C) for 48 h. Cell viability (%) was determined using the WST-8 assay. $*p < 0.05$ indicates statistical significance. **d** LNCaP cells were cultured with (closed symbols) or without (open symbols) of 10 μg/ml poly(I:C) for 48 h in the presence of bafilomycin or chloroquine. Cell viability (%) was determined using the WST-8 assay.

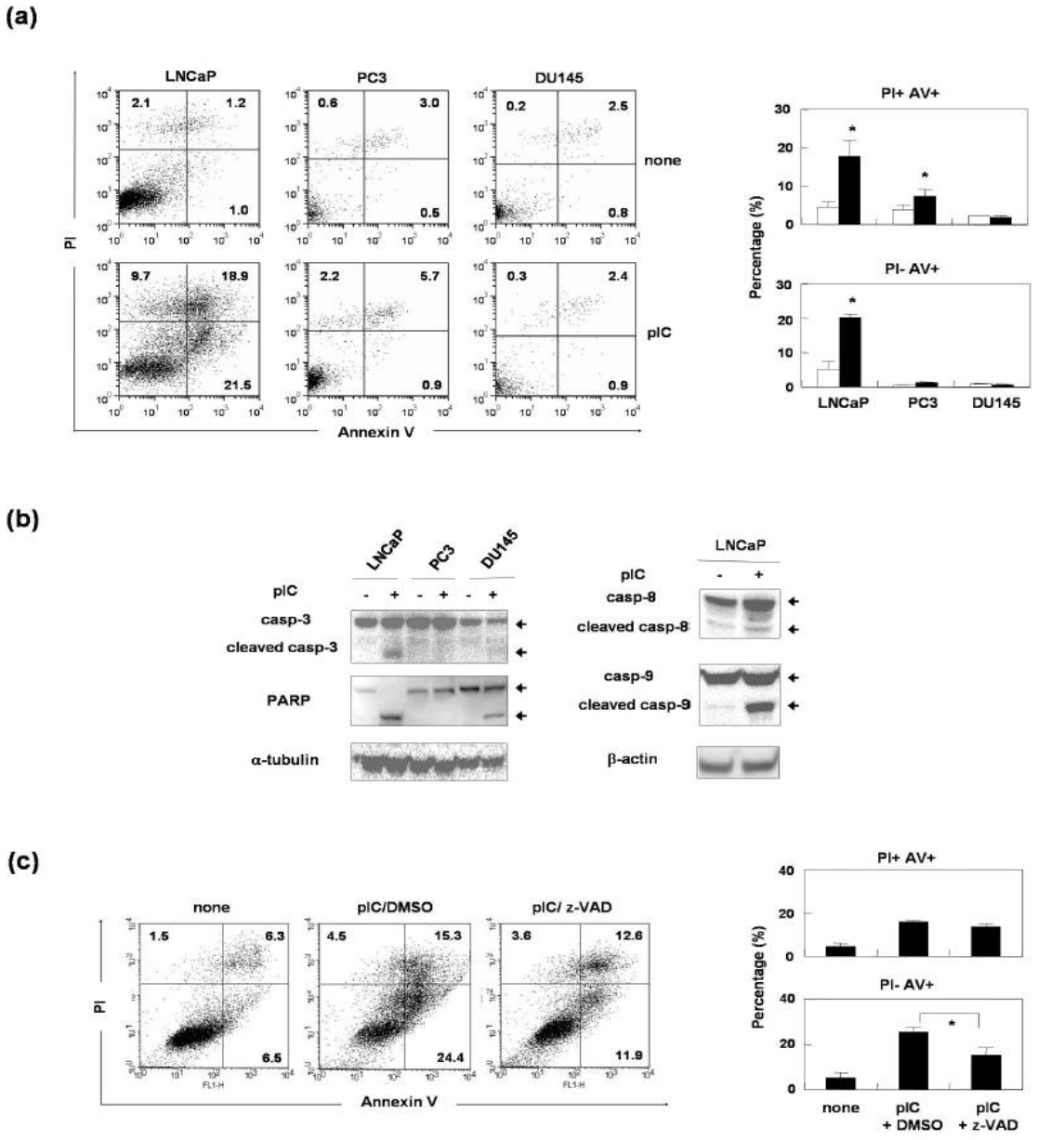

Fig. 2 Caspase-dependent apoptosis of poly(I:C)-treated LNCaP cells. **a** LNCaP cells were cultured with (closed symbols) or without (open symbols) 10 μg/ml poly(I:C) for 48 h, and flow cytometry analysis was performed. Numbers represent the percentage of cells in each subset. The histogram represents a summary of results from three flow cytometry experiments. *$p < 0.05$ indicates statistical significance. AV, Annexin V. pIC, poly(I:C). **b** Protein expression was assessed in cancer cell lines cultured with or without 10 μg/ml

poly(I:C) for 48 h. **c** LNCaP cells were cultured with or without 10 μg/ml poly(I:C) for 48 h in the presence of DMSO or 20 μM z-VAD, and flow cytometry analysis was performed. Numbers represent the percentage of cells in each subset. The histogram represents a summary of results from three independent data. $*p < 0.05$ indicates statistical significance.

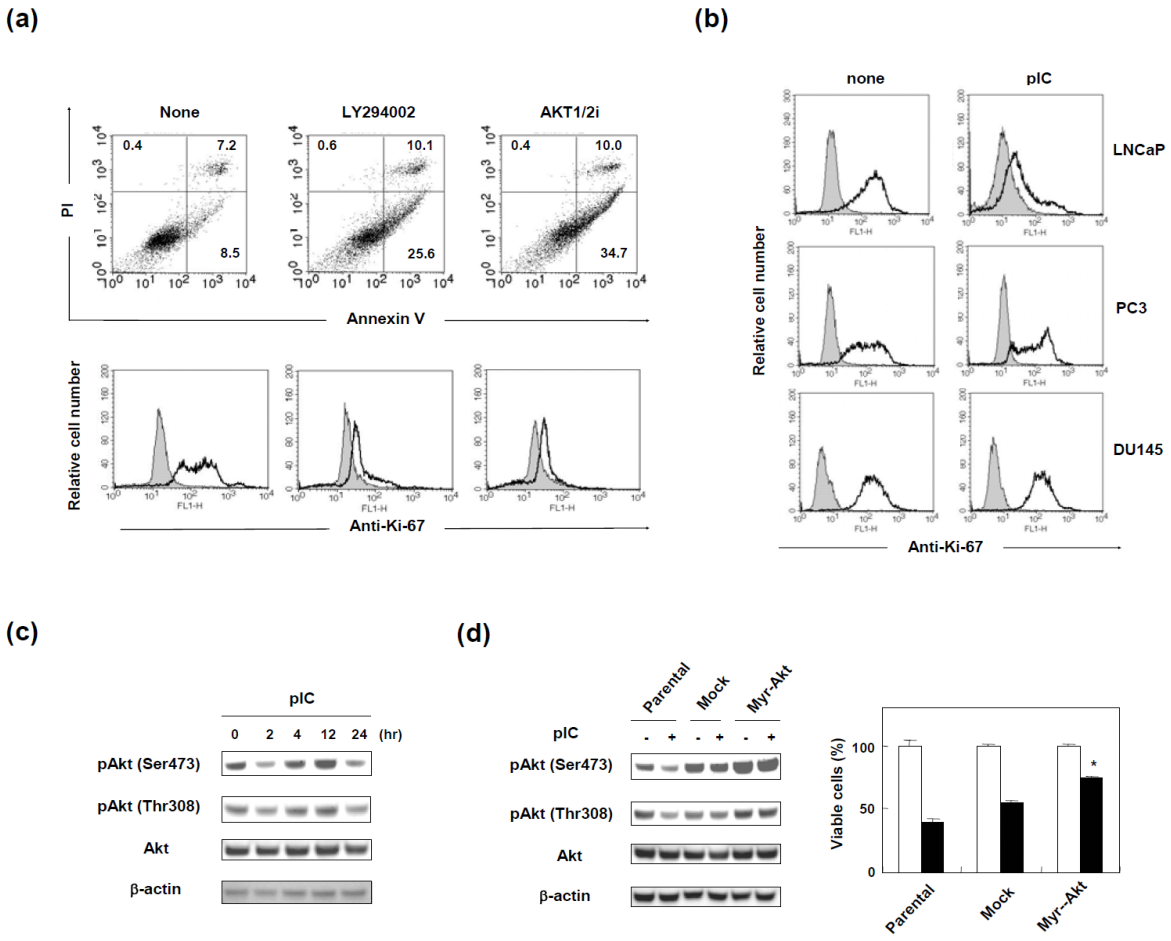

Fig. 3 Participation of the PI3K/Akt pathway in poly(I:C)-induced antitumor effects on LNCaP cells. **a** LNCaP cells were cultured with LY294002 (2 μM: PI3K/Akt inhibitor) and AKT1/2i (1 μM: Akt inhibitor) for 48 h and flow cytometry analysis was performed after Annexin V/PI staining (top) or staining with FITC-conjugated anti-Ki-67 antibodies (bottom). Numbers represent the percentage of cells in each subset and the gray shadow represents staining with an isotype-matched control antibody. **b** Three cell lines were

cultured with or without 10 μg/ml poly(I:C) for 48 h and stained with FITC-conjugated anti-Ki-67 antibodies. pIC, poly(I:C). **c** Protein expression of total Akt and phospho-Akt were assessed in LNCaP cells cultured with 20 μg/ml poly(I:C). **d** (left) parental, mock-, and myr-Akt-LNCaP cells were cultured with or without 20 μg/ml poly(I:C) for 24 h, and immunobot was performed; (right) these three cell lines were cultured with the indicated doses of poly(I:C) and cell viability (%) was determined using the WST-8 assay. *$p < 0.05$ indicates statistical significance.

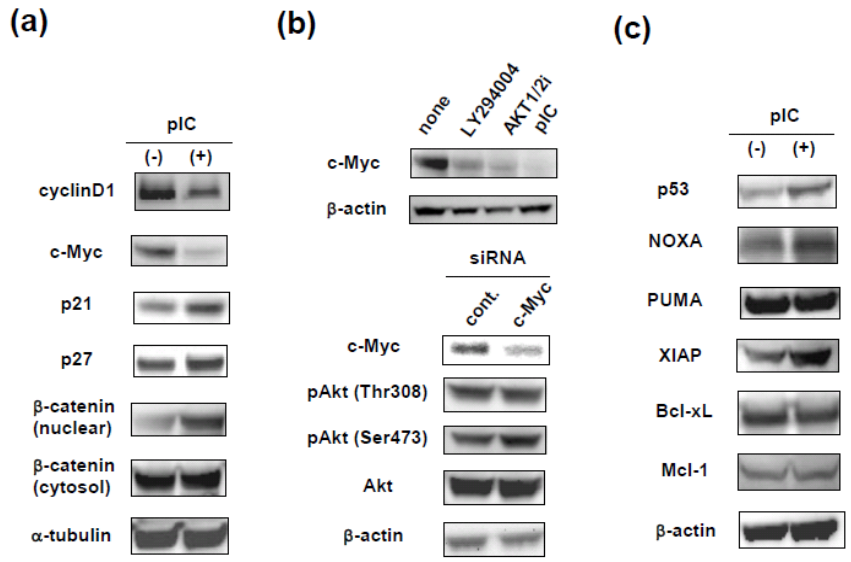

Fig. 4 Changes in proliferation- or apoptosis-related protein levels in poly(I:C)-treated LNCaP cells. **a** Protein expression was analyzed in LNCaP cells that were cultured with or without 20 μg/ml poly(I:C). pIC, poly(I:C). **b** (top) Protein expression of c-Myc in LNCaP cells that were cultured with 2 μM LY294004, 1 μM AKT1/2i, or 20 μg/ml poly(I:C); (bottom) protein expression of c-Myc, total Akt, and phospho-Akt (Thr308 and Ser473) in LNCaP cells that were pre-transfected with c-Myc siRNA were examined. **c** Protein expression was analyzed in LNCaP cells cultured with or without 20 μg/ml poly(I:C).

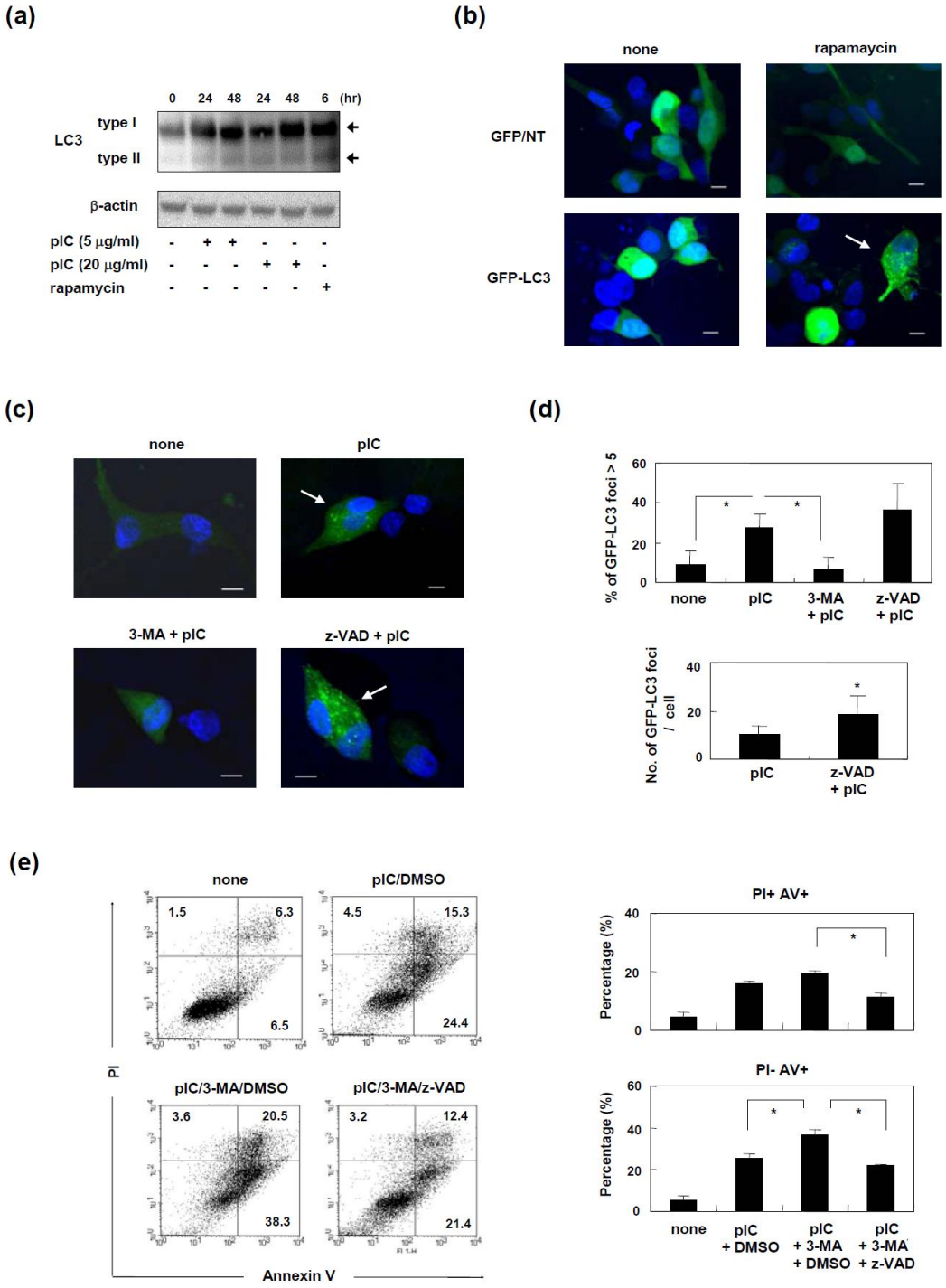

Fig. 5 Autophagy in poly(I:C)-treated LNCaP cells. **a** LNCaP cells were cultured with poly(I:C), or with 2 nM rapamycin for 6 h in the presence of 10 μg/ml pepstatin A, and subjected to immunoblotting analysis. pIC; poly(I:C). **b** LNCaP cells, transfected with the

plasmid encoding GFP-LC3 or GFP/NT, were cultured with or without 2 nM rapamycin for 48 h. Expression of LC3 (green) and nuclear staining with Hoechst 33342 (blue) were observed by confocal microscopy. The arrow shows the GFP-LC3 foci-expressing representative cell. Bar, 10 μm. **c** LNCaP cells transfected with the GFP-LC3 plasmid were cultured with 5 μg/ml poly(I:C) for 48 h in the presence of either 2 mM 3-MA or 20 μM z-VAD. The arrows show the GFP-LC3 foci-expressing representative cells. Bar, 10 μm. **d** (top) the positive percentages of GFP-LC3 foci (>5)-positive cells in total GFP^+ cells are shown; (bottom) the numbers of GFP-LC3 foci in 20 LNCaP cells, which were positive for GFP-LC3 foci (>5), were counted and the means ± SD are shown. Similar results were obtained in two experiments. *$p < 0.05$ indicates statistical significance. **e** LNCaP cells were cultured with or without 10 μg/ml poly(I:C) for 48 h in the presence of DMSO or 20 μM z-VAD and/or 2 mM 3-MA, and flow cytometry analysis was performed. Numbers represent the percentage of cells in each subset. The histogram represents a summary of results from three independent data sets. *$p < 0.05$ indicates statistical significance.