Evaluation of Synergistic Effect of L-Arginine on the Anticancer Activity of Doxorubicin by Using Co-culture System

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Abstract

In the early stage of tumor development, tumor associated macrophage (TAM) works to suppress tumor growth by secreting soluble factors including nitric oxide (NO). L-Arginine (Arg) is a substrate of nitric oxide synthase (NOS) expressed in TAM. Here we examined whether NO produced from Arg by macrophages works to enhance the effect of the anti-cancer drug, doxorubicin (Dox) by using co-culture system of cancer cells with macrophages. By employing colorimetric analyses methods (Griess Reagent and Cell Counting kit-8), we found that NO produced from Arg by co-cultured macrophages could enhance the cytotoxic effect of Dox to cancer cells. Moreover, we found that the augmentation is affected by the order of the addition of Arg and Dox. Prior addition of Arg to Dox and simultaneous addition showed the same enhancement effect, but prior addition of Dox to Arg abolished the augmentation. This suggests that the co-administration of Arg with Dox would be effective treatment to improve chemo-therapies.

Keywords: L-arginine, Nitric oxide, Doxorubicin, Cancer therapy
Introduction

Macrophages constitute a large portion (up to 50%) of tumor mass and these macrophages are called tumor associated macrophages (TAM).\textsuperscript{1, 2} TAM changes their phenotype with the stage of cancer development. In the cancer-initiation stage, TAM is in a type I phenotype (M1) and has anti-tumor activity. With the development of cancer, TAM is converted to type II phenotype (M2) which promotes the tumor development.\textsuperscript{1} Anti-tumor effect of M1 results from the secretion of soluble factors such as nitric oxide (NO), reactive oxygen species, and inflammatory cytokines.\textsuperscript{3, 4} NO is produced by one of the isoforms of nitric oxide synthase (NOS), inducible NOS (iNOS) expressed in M1. NO secreted from M1 shows anti-tumor effect due to the cytotoxicity of reactive nitrogen species derived from NO as well as inhibition of the anti-apoptotic survival signaling.\textsuperscript{5}

L-Arginine (Arg) is the substrate of iNOS to produce NO in M1\textsuperscript{6, 7} and NO production is known to increase by increasing the Arg concentration in the outer media.\textsuperscript{9} Thus, it can be hypothesized that the Arg supplementation would suppress the tumorigenesis. Two research groups confirmed the suppression of the tumorigenesis by the Arg supplementation both in animal models\textsuperscript{10} and human clinical trial.\textsuperscript{11} Recently, polyarginine-containing nanoparticles was reported to retard the tumor growth in animal models.\textsuperscript{7}

We examined whether the sensitivity of cancer cells to anticancer drugs is enhanced by the macrophage mediated NO release from Arg (Fig. 1A). NO has been reported to raise the sensitivity of cancer cells toward anti-cancer drugs such as cisplatin.\textsuperscript{12, 13} This sensitization in cancer cells was attributed to the inhibition of the anti-apoptotic survival signaling by NO.\textsuperscript{14} The combination of NO donors and anti-cancer drugs has been examined in animal studies\textsuperscript{13} and clinical trials\textsuperscript{15, 16} and positive results were observed in some of the studies. In these studies, synthetic NO donors such as NONOates and nitrate esters have been used. These molecules release NO spontaneously or enzymatically without the help of TAM, thereby rapidly raise the concentration of NO. The rapid release of NO would show higher anti-cancer effect whereas it has a risk of systemic hypotension.\textsuperscript{17} In this sense, Arg is a mild NO donor without the side effect.

Jiang et al. recently reported synergetic effect of anti-cancer activity of Arg and Dox by using Dox-loaded dendrimers chemically modified with Arg both in vitro and in vivo.\textsuperscript{8} In their formulation, Arg would be released from the dendrimer by enzymatic reaction in macrophage after endocytotic uptake and iNOS would contribute to the NO production, although it was remained to be examined. The purpose of the present research is to clarify the contribution of Arg and iNOS in the synergy of anti-cancer effect and obtain more insight for future development for co-administration formulation. Thus, we simply used Arg as NO donor, and Arg and Dox were separately added to the co-culture system of macrophage and cancer cells (Fig. 1B). This co-culture system was originally reported by Soma et al to see the effect of NO produced from macrophage on the anti-cancer effect of Dox.\textsuperscript{18}
Experimental

Materials and reagents

L-arginine hydrochloride, doxorubicin, Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), lipopolysaccharides (LPS) from Escherichia coli were all purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Polycarbonate cell culture inserts in multi-dishes were obtained from Thermo Scientific™ Nunc™ (MA, U.S.A). Trypsin - EDTA (0.25%) and L-arginine deficient SILAC Dulbecco's modified Eagle's medium (DMEM) were obtained from Thermo Scientific (MA, USA).

Cell line

Murine macrophage cell RAW 264.7, A549 human pulmonary adenocarcinoma cell, CT-26 murine colon adenocarcinoma cell, and B16 mouse melanoma cell were all obtained from ATCC (Manassas, USA) and cultured in DMEM from ATCC (Manassas, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, USA). The cells were cultured in a humidified atmosphere containing 5% CO₂ in air at 37 °C.

Measurement of nitrite oxide release

Macrophages RAW 264.7 or cancer cells were seeded into 24-well plate (50000 cells/well) and incubated for 24 h. Then the medium was removed and cells were incubated with fresh L-arginine deficient medium with or without the stimulation of LPS (1 μg/mL) for 6 h. After that, cells were incubated with various concentrations of Arg for another 48h. The supernatant of the cultured medium was collected and centrifugated at 1500×g for 15 min. The amount of NO which reflected by nitrite was quantified by using the Griess Reagent Kit (Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. The absorbance was measured at 540 nm using Infinite M Plex microplate reader (TECAN, Switzerland).

Measurement of the inhibition of cell growth

The experiments were conducted in polycarbonate cell culture inserts which consisted of two compartments separated by a porous membrane (0.4 μm pore size, catalog number 140620, Thermo Scientific Nunc). Cancer cells (A549, B16, CT-26) were cultured in 50000 cells/well at the lower chamber and RAW 264.7 were seeded into the upper chamber in 80000 cells/well. After 24 h of culture at 37 °C, LPS (1 μg/mL) and L-NAME (2 mM) were added to RAW 264.7 in the upper chamber and incubated for 6 h. Then various Arg and Dox were added in the upper chamber and incubated for 48 h. After separating RAW 264.7 and the cancer cells, the medium was removed and cells were incubated with fresh medium containing Cell Counting kit-8 reagent (Dojindo, Kumamoto, Japan) for another 4 h. The absorbance was measured at 450 nm by using Infinite M Plex microplate reader (TECAN, Switzerland).
Statistical analysis

All data are expressed as mean ± SD. Data were evaluated using one-way analysis of variance and statistical analysis was performed via the GraphPad Prism software. A value of $p \leq 0.05$ was considered significant.
Results and Discussion

NO production from Arg in macrophage and cancer cells

NO production ability of macrophage and cancer cells from Arg in response to LPS stimulation was examined. First, the cells were stimulated with LPS (1 μg/mL) for 6 h to induce the expression of iNOS.19 LPS-stimulated cells were regarded as a model of M1 cells.20 Then, the cells were incubated in the presence of Arg for 48 h. The produced NO was detected by Griese reagent, which detects NO\textsubscript{2} in media derived from the oxidization of NO.21 As shown in Fig. 2, Raw 264.7 macrophage produced NO with the increase of Arg concentration, which is consistent with previous reports that exogenous Arg could produce NO in the activated macrophages.9 In contrast, NO production was not increased in all three cancer cell lines even at higher concentrations of Arg. It was reported that for the induction of iNOS in cancer cells, inflammatory cytokines were required in addition to LPS.22 The obtained results confirmed that macrophage can be activated by simple stimulation and can be a source of NO by using Arg as a substrate.

Arg augmented cytotoxicity of Dox to cancer cell

We examined the effect of NO production of macrophages on the cytotoxicity of Dox toward co-cultured cancer cells, which imitates the tumor environment (Fig. 1B). The cancer cells were cultured in the lower chamber. As shown in Fig. 3, co-culturing with LPS-activated macrophages reduced around 30% in the viability of the three kinds of cancer cells. This reduction of the viability resulted from secreted soluble factors from the macrophages. A similar level of viability reduction in cancer cells was reported previously in the co-culture system.23 When Dox alone was added to the macrophages in the upper chamber, the viability of the three cancer cell lines was further reduced. Because the macrophages were cultured in high density in the upper chamber, the free diffusion of Dox through the separating membrane would be blocked.18 Thus, the further reduction of the cancer cells by Dox indicated the penetration of Dox through the macrophage to reach the cancer cells in the lower chamber. In contrast, the addition of Arg alone in the upper chamber did not affect the viability of the cancer cells, indicating that the NO concentration produced from the macrophages was mild and would not induce cytotoxicity. However, the simultaneous addition of Dox with Arg in the upper chamber further reduced the viability of the three cancer cells comparing with those treated with Dox alone, indicating a synergetic effect in combination of Dox and Arg. It is notable that the co-cultured macrophages tolerated the addition of Dox and/or Arg probably due to a lower sensitivity of macrophages to Dox (Fig. 3).

To examine the contribution of NO synthesis from Arg in the enhancement of the cytotoxicity of Dox, NOS inhibitor L-NAME was added to the coculture system. As shown in Fig. 4, inhibition of NOS abrogated the synergetic effect of Dox with Arg. The mechanism of this tumoricidal effect augmentation probably due to the
increased sensitization of cancer cells to Dox by NO.\textsuperscript{24, 25} Meanwhile, inhibition of DNA repair by the reactive nitrogen oxide species derived from NO may also contribute the drug efficacy augmentation.\textsuperscript{26} It has been reported that some developed analytical methodologies could quantify the anticancer drugs of cell lines which might help to further clarify the NO's function.\textsuperscript{27}

\textit{The sequence in the addition of Dox and Arg is critical for the augmentation in the Dox effect}

For the application of the present synergy to the therapy, we should consider the timing of the administration of Dox and Arg. We assessed the effect of the sequence of the addition of Dox and Arg in the upper chamber. As shown in Fig. 5, a sequence of Arg-first (6 h prior to Dox) showed a similar augmentation effect with the simultaneous addition of Arg and Dox. However, the opposite sequence (Dox-first; 6 h prior to Arg) abolished the augmentation effect. The disappearance of the synergetic effect would be explained by the reported fact that Dox deteriorates the macrophage’s ability to produce NO,\textsuperscript{18} which will reduce total amount NO released from the macrophages. However, some reported advanced biosensor analytical technologies which are able to monitor cells’ response to a certain surrounding environment might give more direct mechanism about this phenomenon.\textsuperscript{28} According to the obtained results, simultaneous or Arg-first would be the suitable timing to use Arg as an enhancer of Dox cytotoxicity.

\textbf{Conclusions}

Here we clarified that NO produced from macrophage by using Arg as an iNOS substrate augmented cytotoxicity of Dox to cancer cells by using co-culture system. This mechanism would be a basis of synergetic anti-cancer effect of Arg and Dox in vivo reported by Jiang et al using Dox-loaded Arg-modified dendrimers.\textsuperscript{8} The present strategy which utilizes TAM as a source of NO would enhance the efficacy of anti-cancer drugs without amplifying their side effects.

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Fig. 1. (A) Schematic illustration of synergy of NO produced from macrophage with anti-cancer drug, doxorubicin. (B) Experimental setup to prove the synergy mimicking the tumor environment.

Fig. 2. NO production from RAW 264.7 macrophage and cancer cells (CT26, A549 and B16). After 6 h incubation with LPS (1 μg/mL), Arg was added to the cells and incubated for another 48 h. Data are expressed as mean ± SD (n = 3). ** p < 0.01, *** p < 0.001 compared with ctrl (without LPS stimulation).
Fig. 3. Synergistic effect of Arg with Dox on the cytotoxicity of (A) CT26, (B) A549, (C) B16 cells (left rows), and co-cultured Raw 264.7 (right rows). After six hours stimulation of Raw264.7 with LPS (1 μg/mL), Arg (1 mM) and Dox (1 μM for CT26 and A549, 1.5 μM for B16) were added to Raw 264.7 in the upper chamber and incubated for another 48 h. Dox concentration was optimized for each cell line to clearly see the synergetic effect between Dox and Arg. Results are expressed as mean
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Fig. 4. Effect of NOS inhibition on the synergistic effect of Arg and Dox in the co-culture system (A) C T26, (B) A549, and (C) B16. The experimental procedures were same with Fig. 3, except for L-NAME (2 mM) was added to Raw 264.7 in the upper chamber together with LPS. Results are expressed as mean ± SD (n = 3). * p < 0.05, ** p < 0.01, N.S.: not significant.
Fig. 5. The sequence of Arg and Dox addition on the synergetic effect. (A) CT26, (B) A549, and (C) B16. Arg → Dox represents Arg was added 6 h prior to Dox; Arg + Dox represents simultaneous addition; Dox → Arg represents Dox was added 6 h prior to Arg. Other experimental conditions were same with Fig. 3. Results are expressed as mean ± SD (n = 3). * p<0.05, ** p<0.01, N.S.: not significant.
**Figure Captions**

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