PEGylated recombinant human arginase as a drug for breast cancer

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KEY MESSAGES

1. rhArg-PEG is highly potent and kills all breast cancer cell lines (including 'triple-negative' and highly aggressive metastatic cells) in vitro and in vivo in a receptor-independent manner.
2. rhArg-PEG induces multiple cancer cell death pathways that are cell-line dependent.
3. rhArg-PEG inhibits mTOR, activates AMPK, and induces a novel type of biphasic autophagic response in cancer cells, leading to autophagic cell death.
4. rhArg-PEG kills cancer cells lacking ornithine transcarbamylase and/or argininosuccinate synthetase and inhibits more tumours than arginine deiminase-PEG does. It is a promising personalised medicine.

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Introduction

Breast cancer is the most common cancer in women and accounts for the highest female cancer death. As of 2008, approximately 207 500 of 1 383 500 breast cancer patients worldwide are resistant to hormone receptor- or HER2-targeting therapies. Safe and effective treatments against 'triple-negative' breast cancer (not expressing oestrogen receptor, progesterone receptor, or HER2) are in demand.

Arginine is essential for the growth of a variety of tumours, including hepatocellular carcinomas (HCCs) and melanomas. Depletion of arginine (using arginine-degrading enzymes or arginine-free growth medium) leads to massive growth retardation, cell cycle arrest, and/or apoptosis in many cancer cell lines.[1-4] In contrast, normal cells arrest at G0 phase under arginine deprivation and resume cycling once arginine is replenished. This selective anti-tumour property makes arginine deprivation a valuable means for cancer treatment. Our laboratory has shown that human hepatic arginase (arginase I), which catabolises arginine to form ornithine and urea, possesses anti-proliferative properties against HCC and melanoma cells in vitro and in vivo. Patients with HCC who underwent transhepatic arterial embolisation exhibited remarkable remission, as a result of the systemic release of endogenous arginase I induced by the treatment. We first reported that a recombinant form of human arginase I (rhArg) is highly potent against HCC and melanoma cell lines.[1,2] Covalent modification of rhArg, using polyethylene glycol (PEG) with a molecular weight of 5000, gives the enzyme a longer half-life without reducing its enzyme activity or anti-tumour efficacy. This PEGylated form of rhArg (rhArg-PEG) is highly effective in inhibiting the growth of HCC and melanoma xenografts in nude mice, and thus is a potential treatment for these cancers.[1,2] Other studies have reported on anti-tumour properties of rhArg and its bioengineered derivatives. rhArg-PEG has been proved to be safe and effective in a phase 1a dose finding and phase 1b efficacy study in 35 advanced liver cancer patients at The University of Hong Kong.[5]

Depletion of arginine using the arginine deiminase (ADI) enzyme is effective against several cancer cell types in vitro and in vivo and is under extensive clinical trials for several cancer types. However, expression of the urea cycle enzyme argininosuccinate synthetase (ASS) in tumour cells can lead to resistance towards ADI treatment by allowing recycling of arginine from its enzymatic product, citrulline (Fig 1). ASS expression is commonly observed in cancer cell lines and tumour biopsies from patients. ADI enhances the sensitivity of MCF-7 towards ionising radiation treatment and kills MDA-MB-231, which is ASS-deficient.[3] Only upon the knockdown of ASS expression using RNAi, was MCF-7 rendered susceptible to ADI-induced growth inhibition, thus proving that ASS expression and ADI resistance of breast cancer are closely linked. Immunohistochemistry revealed that >90% of the breast cancer biopsies sampled were ASS-positive, which could be resistant towards ADI treatment. Arginase is effective against breast cancers, because ASS expression alone cannot

render tumour cells resistant to its inhibitory effects, as shown in our study on ASS-expressing HCC cell lines.[1] Only cancer cells that simultaneously express the urea cycle enzymes of ASS, argininosuccinate lyase, and ornithine transcarbamylase were resistant to arginase treatment, whereas the co-expression of the first two could lead to ADI resistance (Fig 1). Arginase, therefore, can potentially inhibit the growth of a larger subset of tumours when compared with ADI. As rhArg-PEG is derived from human arginase, it should be a safer drug with less immunogenicity problems, compared with the bacterial enzyme ADI.

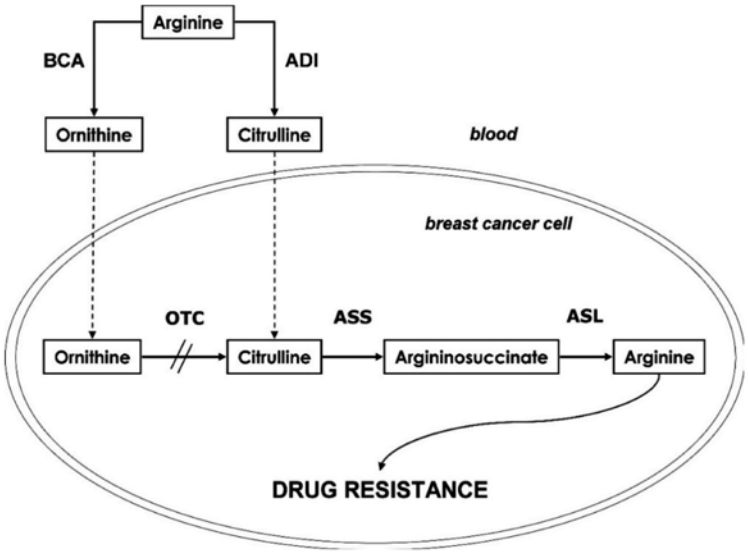

FIG 1. Schematic diagram showing the model relating sensitivity of tumour cells to arginine deprivation with either the Bacillus caldovelox arginase (BCA) or the bacterial arginine deiminase (ADI). Cells that express both argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) are resistant to ADI but remain BCA-sensitive.

Methods

MDA-MB-231 human breast tumour cell xenograft mouse model

Female nude mice aged 5 to 6 weeks were used. They were kept in groups of five per cage and provided food and water ad libitum. MDA-MB-231 cells were harvested by trypsinisation and washed twice with 1× phosphate-buffered saline (PBS). The cell number was counted by trypan blue method. 1 $\times 10^7$ cells of MDA-MB-231 were resuspended in 100 µL PBS-matrigel mixture (with a ratio of 1:1) and injected subcutaneously into the right flank of the mice. The tumour growth was monitored by measuring length and width of the tumour by digital caliper regularly. The estimated tumour volume was calculated by the equation: Tumour volume (mm^3) = (length × width^2)/2. When the tumour volume grew to around 500 mm^3, the mice were sacrificed and dissected. Tumours were collected and excised to around 10 mm^3 each. Each small tumour was transplanted into a new female nude mouse after anaesthesia by ketamine and xylazine. The dissected tumours were transplanted to the right flank of the mice. For MDA-MB-231 xenograft studies, 40 female nude mice transplanted with MDA-MB-231 solid tumours were used. The mice were separated into four groups randomly when the tumour volume reached an average of 50 mm^3. Mice in the control group were injected with 1× PBS once a week; mice in the rhArg-PEG group were injected with 500 U rhArg-PEG (290 µL, 2 mg) once a week; mice in the chloroquine (CQ) group were injected with 40 mg/kg CQ once every 2 days; and mice in the combination group (rhArg-PEG plus CQ) were injected with both 500 U rhArg-PEG once a week and 40 mg/kg CQ once every 2 days. The two drugs were injected separately with a 4-hour gap when both rhArg-PEG and CQ were injected to the

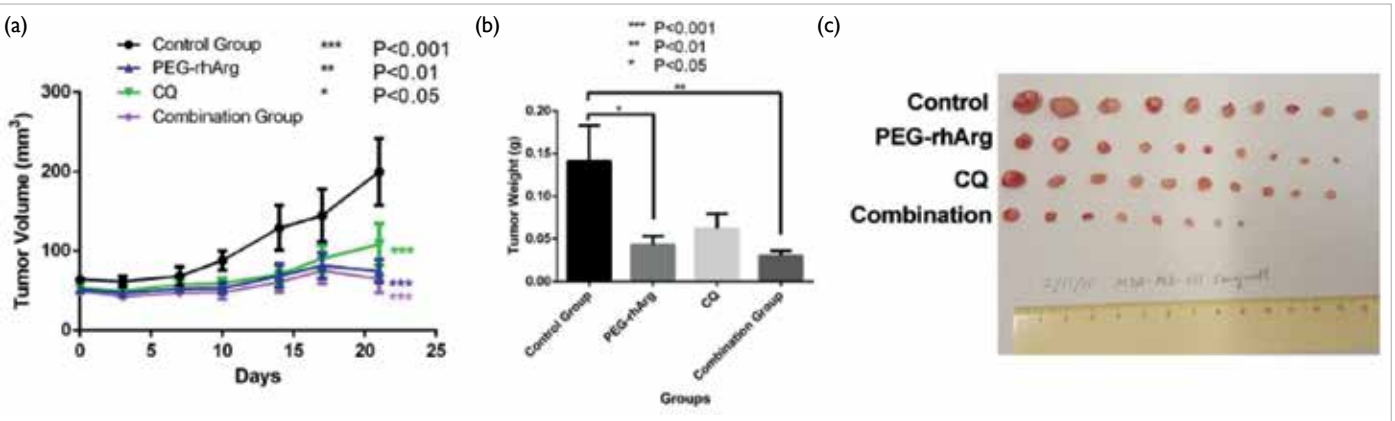

FIG 2. Anti-tumour effects of rhArg-PEG, chloroquine (CQ), and combination of both on MDA-MB-231 xenograft model in terms of (a) tumour volume, (b) tumour weight at the end of the experiment, and (c) tumour appearance.

mice within the same day. During the experiment, blood samples were collected from the saphenous vein. Tumour volume and the weight of mice were monitored regularly. The mice were then sacrificed and dissected to collect and weigh the tumours.

4T1 metastatic cell allograft mouse model

For 4T1 allograft studies, female Balb/c nude mice (5-6 weeks of age) were used. They were kept in groups of five per cage and provided food and water ad libitum. 4T1 cells were harvested by trypsinisation and washed twice with 1× PBS. The cell number was counted by the trypan blue method. Cells (1×10^5) of 4T1 were resuspended in 100 µL 1× PBS and injected subcutaneously into the right flank. Mice were separated into two groups by random when the tumour had a mean length of 8 mm after 11 days of inoculation. Mice in the control group were injected with 1× PBS weekly. Mice in the rhArg-PEG group were injected with 500 U (2.4 mg, 400 µL) rhArg-PEG weekly. Blood samples were collected from the saphenous vein before injecting drug/PBS. The amino acid content of the serum was analysed by amino acid analyser (Biochrome). Tumour size and the weight of mice were monitored regularly. The mice were then sacrificed and dissected to collect and weigh the tumours. Lungs were also collected and washed with 1× PBS and fixed in Bouin solution overnight and then washed with 1× PBS. Lung metastatic nodules were counted.

Statistical analysis

Statistical analyses were performed using Student's *t*-test, Mann-Whitney *U* test, or analysis of variance. For multiple comparisons after analysis of variance, the Tukey post hoc pairwise comparison approach was used. A P value of <0.05 was considered significantly different.

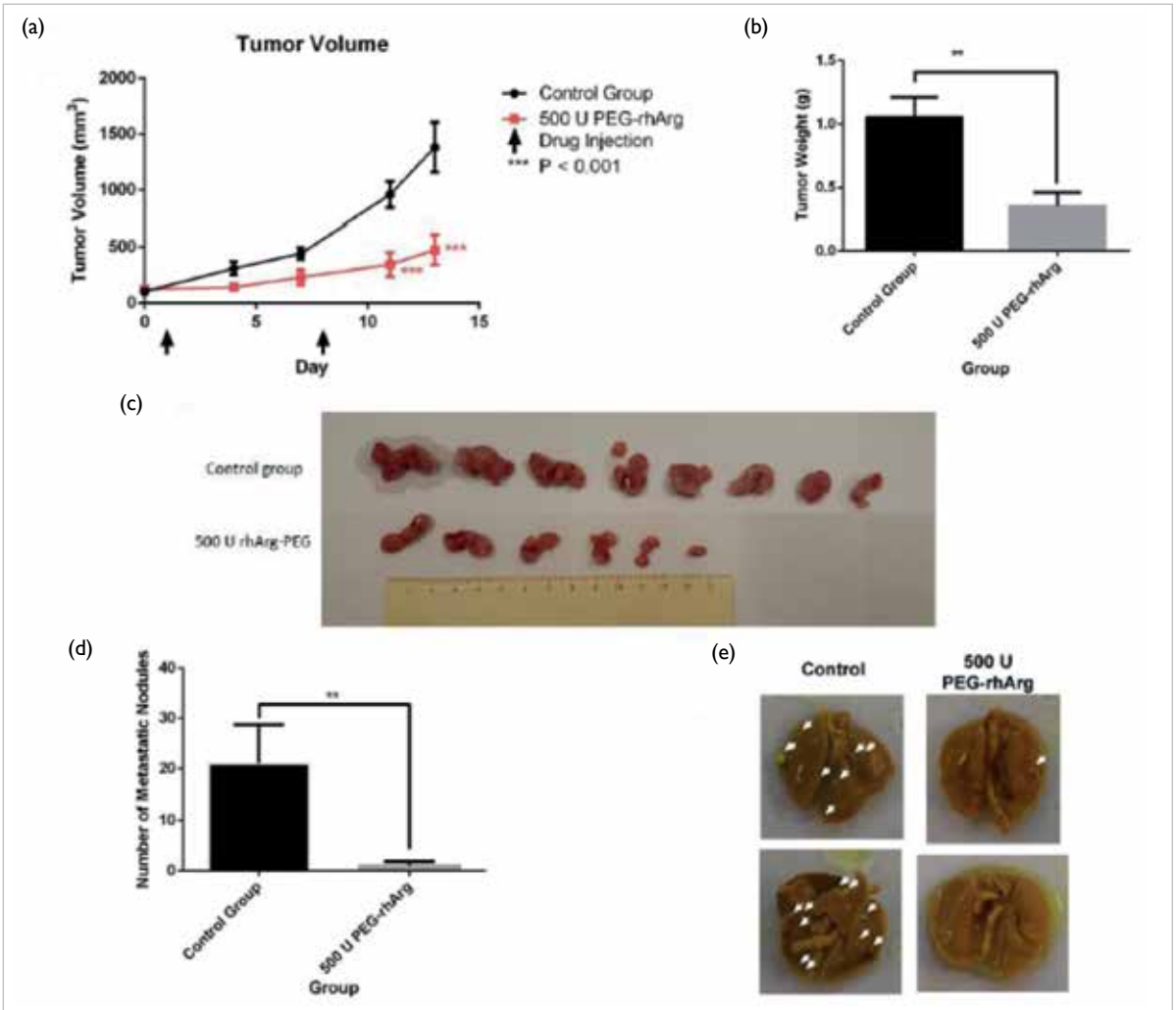

FIG 3. Effects of intraperitoneal injection of 500 U rhArg-PEG once per week on the 4T1 cell allografts in control and rhArg-PEG groups in terms of (a) tumour volume, (b) tumour weight at the end of the experiment, (c) tumour appearance, (d) number of metastatic nodules in the lungs, and (e) relatively large nodules on the lung surface (arrows).

Results

rhArg-PEG inhibits tumour growth in the MDA-MB-231 xenograft model

Chloroquine can bind with lysosome and prevents endosomal acidification. It can prevent degradation of autophagosomes and thus inhibit autophagy. In vitro data showed that CQ caused synergistic effects against MDA-MB-231 when it was combined with rhArg. Injecting 500 U of rhArg-PEG once per week significantly inhibited the growth of MDA-MB-231 xenograft (Fig 2). We tested whether CQ had synergistic effects when combined with rhArg-PEG. CQ alone inhibited the growth of the tumour, but it did not significantly improve efficacy of rhArg-PEG when combined with rhArg-PEG, consistent with data from Qiu et al.[3]

rhArg-PEG inhibits tumour growth and metastasis in the 4T1 cell allograft model

The efficacy of rhArg-PEG was tested in the 4T1 cell allograft metastasis mouse model. A dosage of 500 U of rhArg-PEG once per week significantly inhibited the growth of 4T1 solid tumour when compared with the control group (PBS group) [Fig 3]. 500 U of rhArg-PEG was sufficient to deplete all the serum arginine in mice for at least 7 days. The growth of the 4T1 allograft was significantly inhibited by the arginine depletion caused by rhArg-PEG. The aggressive 4T1 cancer cells have great potential to metastasise to distant organs. The lungs of the mice were evaluated for the anti-metastatic effects of rhArg-PEG (Fig 3). All mice in the control group of the 4T1 allograft contained the metastatic nodules in their lungs, whereas only 25% of the mice in the rhArg-PEG treatment group contained visible metastatic nodules. rhArg-PEG significantly reduced the number and the size of metastatic nodules.

Discussion

In our study, rhArg-PEG inhibited the tumour growth in the 'triple-negative' MDA-MB-231 xenograft model. PEGylation extended the serum half-life of protein drugs and reduced their immunogenicity. rhArg-PEG did not show immunogenicity or neutralising antibody problems. rhArg-PEG depleted serum arginine level, although some anti-drug antibodies were detected. Arginine depletion by 500 U rhArg-PEG injected once per week inhibited the growth of 4T1 tumour cells in nude mice significantly and the drug was tolerable.

In addition, arginine starvation by rhArg-PEG inhibited the 4T1 tumour cell metastasis. Arginine is a precursor of polyamines, which are important for formation of lamellipodia and stress fibres during cell migration. Arginine plays an important role in cell migration and tumour metastasis. Depletion of arginine by rhArg-PEG may inhibit the production of polyamines and therefore inhibit metastasis of the aggressive 4T1 breast cancer cells in the mouse model. As cancer metastasis is the major cause of death, our finding may open up new possibilities for a more effective cancer therapy.

Conclusion

rhArg-PEG is safe and effective treatment against 'triple-negative' or highly metastatic breast cancer. rhArg-PEG is derived from human arginase and should be a safer drug with less immunogenicity problems, compared with bacterial enzyme ADI.

Acknowledgement

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