Selective Tumor Imaging by a Novel Tumor Specific Aralin-Infrared-to-Visible Phosphor Conjugate

Y Kawasaki1,2, Y Gotoh1,2, K Tokuzen2,3, M Kamimura,4,5, T Komeno1, M Tomatsu6, R Todoroki1,2, Y Nagasaki2,4,5, K Soga2,3 and F Tashiro1,2

1 Department of Biological Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, 278-8510 Chiba, Japan
2 Polyscale Technology Research Center, 2641 Yamazaki, Noda, 278-8510 Chiba, Japan
3 Department of Materials Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, 278-8510 Chiba, Japan
4 Graduate School of Pure and Applied Science, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, 305-8573, Ibaraki, Japan
5 Tsukuba Research Center for Interdisciplinary Materials Science (TIMS), 1-1-1 Tennoudai, Tsukuba, 305-8573, Ibaraki, Japan
6 Akita Research Institute for Food and Brewing (ARIF), 4-26 Azasanuki Arayamachi, Akita, 010-1623 Akita, Japan

E-mail: ftashir@rs.noda.tus.ac.jp

Abstract. Aralin is a novel cytotoxic protein from Aralia elata and selectively induces apoptosis in transformed cells as compared to normal cells (1). Aralin is a lectin specific for sugar chain such as galactose and possesses RNA N-glycosidase activity. In this study, antitumor potency of aralin was analyzed using the poly(ethyleneglycol) (PEG)/streptavidin co-immobilized infrared-to-visible upconversion phosphors, Y2O3 nanoparticles (2). Cy3-conjugated aralin could clearly detect the surface of SV40-transformed VA13 and human cervical carcinoma HeLa cells, but to a lesser extent on the normal human fibroblast WI-38 cells. Conjugation of aralin with PEGylated Y2O3 nanophosphor was carried out via biotin-avidin binding. The Y2O3-conjugated aralin also clearly visualize by a fluorescence microscope measurements equipped with near-infrared excitation source scanning in HeLa cells. It is also important to note that no remarkable damage to the cells was observed during these observations. Thus, these data imply that the Y2O3-conjugated aralin would potentially be useful material for tumor detection in vivo.
1. Introduction
Visualization of the tumor cells is one of the most important techniques to find out cancer patient in early stage of the disease. We have been reported that the novel type II ribosome-inactivating protein aralin from Aralia elata is an anti-tumor cytotoxic protein. Aralin is composed of A- and B-chains, whose N-terminal sequences show homologies with nigrin A-chain and ricin B-chain, respectively, and highly toxic to tumor cell lines compared with normal cell lines. Since aralin possesses a lectin-like specificity for galactose, lactose and fucose, it seems that aralin recognizes some types of tumor cell surfaces, in which sugar-added receptors are expressed to facilitate the delivery of toxic A-chain by endocytosis. After the entry into the cytosol, the A-chain inactivates 60S ribosomal subunits by catalyzing specific RNA-N-glycosidase activity and inhibited protein synthesis. Therefore, aralin may prove to be a suitable immunotoxin for tumor therapy and detection.

In order to visualize such specific toxicity against cells and molecular events in the cytosol, fluorescence bioimaging system is one of the most powerful tools. However, ultraviolet light causes toxic effect to living organism including cells. If near infrared light can be utilized for this system, the bioimaging technique will be improved. This paper communicates biolabeling of aralin by novel PEGylated nanophosphors, which can be excited by near infrared laser. The conjugate thus obtained was applied for an in vitro experiment to confirm antitumor potency.

2. Experimental Section

2.1. Cytotoxicity assay
Normal and cancer cell lines were seeded at 1 x 10^4 cells/well in 24-well plate and cultured in Eagle’s minimal essential medium (MEM) or Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS for 24 h. Various concentration of aralin were added to cells for 36 h and cytotoxicity of aralin was examined by trypan blue dye exclusion assay.

2.2. Preparation of Cy3-conjugated aralin and detection of cell surface receptor
The N-terminal amino groups of aralin were labeled with Cy3 (GE healthcare, Piscataway, NJ, USA) according to the manufacture’s procedures. Free Cy3 was removed by Sephadex G-50 gel filtration. Cells attached to Lab-Tek II chamber slides (Nalge Nunc International, Naperville, USA) and suspended in MEM with 10% FCS were incubated with 3.2 x 10^-9 M Cy3-aralin at 4°C for 15 min and the cells were examined under a fluorescence microscope (Axioplan 2, Carl Zeiss, Oberkochen, Germany).

2.3. Preparation of PEGylated nanophosphor conjugated aralin and in vitro experiments
The N-terminal amino groups of aralin were labeled with biotin labeling kit-NH2 (Dojindo, Kumamoto, Japan) according to the manufacture’s procedures. The PEG/streptavidin modified infrared-to-visible upconversion phosphors, Y2O3 nanoparticles were prepared as previously described. Two hundred ng biotin-conjugated aralin and 1 µg PEG/streptavidin-Y2O3 were reacted at 4°C for 30 min. VA-13 cells were suspended with HEPES buffer (20 mM Hepes-KOH, pH7.5, 150 mM NaCl and 3% BSA) and incubated with biotin-aralin and PEG/streptavidin-Y2O3 at 4°C for 2 h. and examined under a fluorescence microscope equipped with near-infrared excitation source (TCLDM9, Thorlabs, Newton, NJ, USA).

3. Results and Discussion

3.1. Cytotoxic activity of aralin
To investigate the effects of aralin on the normal human fibroblasts WI-38 and cancer cells such as tumorigenic simian virus 40 large T antigen-transformed WI-38 cells (VA-13) and human cervical carcinoma HeLa cells, the cell viability was assayed by trypan blue dye exclusion procedure. Aralin
exerted a potent cytotoxicity against human cancer cells (VA-13, HeLa) compared with WI-38 cells (Figure 1). The IC\textsubscript{50} values of aralin against WI-38, VA-13 and HeLa cells were 42.3, 4.0 and 1.6 ng/ml, respectively.

3.2. Aralin binding with cell surface
The antitumor potency of aralin was then investigated using Cy3-conjugated aralin. When WI-38, VA-13 and HeLa cells were incubated with 3.2 x 10\textsuperscript{-9} M Cy3-aralin at 4°C for 15 min, the cell surface of VA-13 and HeLa cells were significantly labeled with Cy3-aralin compared with WI-38 cells, but not by Cy3-BSA (Figure 2). Fluorescence of Cy3-aralin was hardly detected in WI-38 cells under the same conditions, but slightly detected for further incubation at 4°C (data not shown). These results suggest that the expression level of the aralin receptor on the cell surface of WI-38 cells and/or its binding affinity are lower than those of VA-13 and HeLa cells.

3.3. Selective tumor imaging using PEGylated nanophosphor conjugated aralin
In order to confirm near-infrared excitation system, PEGylated Y\textsubscript{2}O\textsubscript{3} nanophosphor-conjugated aralin was investigated for the potent tumor imaging. VA-13 cells were incubated with 3.2 x 10\textsuperscript{-8} M PEGylated nanophosphor conjugated-aralin or BSA at 4°C for 2 h and examined under a fluorescence microscope equipped with near-infrared

**Figure 1.** Cytotoxic activity of aralin in normal and various human tumor cells. Normal and cancer cell lines were seeded at 1 x 10\textsuperscript{4} cells/well in 24 well plate and cultured in MEM or DMEM supplemented with 10% FCS for 24 h. Various concentration of aralin were added to cells for 48 h and cytotoxicity of aralin was examined using trypan blue dye exclusion assay.

**Figure 2.** Detection of aralin cell surface receptor. WI-38, VA-13 and HeLa cells were incubated with 3.2 x 10\textsuperscript{-9} M Cy3-conjugated aralin or BSA at 4°C for 15 min. The cells were observed under a fluorescence microscope.
As shown in Figure 3, $Y_2O_3$ excitation was detected with PEGylated nanophosphor conjugated-aralin treated on cell surface, but not by PEGylated nanophosphor conjugated-BSA. Therefore, we achieved detection of tumor cells using anti-tumor protein and near infrared excitation phosphors $Y_2O_3$. It should be noted that the conjugate was excited by 980 nm laser (200 mW), thus no cell damage was observed in this study.

**Figure 3.** Selective tumor imaging using biotin-aralin and PEG/streptavidine-$Y_2O_3$

VA-13 cells were incubated with $3.2 \times 10^{-6}$ M biotin-aralin or BSA at 4°C for 120 min. VA13 cells were applied on slides glasses and examined under a fluorescence microscope equipped with a 980-nm laser diode for the near-infrared excitation source.

4. **Conclusion**

These results indicate that the selective tumor cells detection systems using PEGylated nanophosphor conjugated-aralin would be expected as useful biolabeling materials for tumor detection in vivo.

5. **Acknowledgements**

This work was partly supported by “Academic Frontier” Project for Private Universities: Matching Fund Subsidy from MEXT (Ministry of Education, Cultures, Sports, Science and Technology), 2006-2010. This work was also supported by KAKENHI(17590098): Grant-in-Aid for Scientific Research (C), 2005-2006.

**References**

[1] Tomatsu M., Kameyama M.O., Shibamoto N. 2003 *Cancer letter* **199** 19
[2] Kamimura M., et. al. 2008 *Langmuir* **24** 8864
[3] Hoffman R.M. 2005 *Nat. rev. cancer* **5** 796
[4] Tomatsu M., et. al. 2004 *Biol. Chem.* **385** 819