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Production and Application of LPA Polyclonal Antibody†

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Abstract—By using colloidal gold as a hapten carrier, a kind of antibody against lysophosphatidic acid (LPA) was developed and used to successfully detect 500 ng/mL LPA in dot immunogold filtration assay. Such application of the LPA antibody could offer us a way to diagnose ovarian cancer at its early stage.

Lysophosphatidic acid (LPA, 1-acyl-2-hydroxy-sn-glycero-3-phosphate) has been known as a precursor of phospholipid biosynthesis for a long time. It has recently emerged as an intercellular signaling molecule that is rapidly produced and released by activated cells, notably platelets, to influence target cells by acting on a specific cell-surface receptor.[1,2,3,4] In 1998, Y. Xu et al. found patients with ovarian cancers had significantly higher plasma LPA levels than those with other cancers, benign gynaecological tumors or no cancer. Furthermore, they found that by using LPA as a biomarker,[5,6] the chance of detecting ovarian cancer at stage I could be remarkably increased. While the 5-year survival rate of women with advance ovarian cancer is less than 15%, the long-term survival rate of patients with stage I ovarian cancer is as high as 90%.

However, the purification and identification of LPA in plasma is complicated and time-consuming.[4,6,7] An immunoassay depending on the antibody will be an easy and fast way for the determination of LPA. In this paper, we produced a polyclonal antibody to LPA using colloidal gold as a macromolecular carrier to enhance the immunogenicity of LPA and developed an immunoassay, dot immunogold filtration, to qualitatively detect LPA. The production of the polyclonal antibody against LPA will be helpful for early diagnosis of ovarian cancer and further studies on biologic functions of LPA in the differentiation and propagation of ovarian cancer cells.

Methods

Production and purification of antisera

LPA (16:0) (Avanti Polar-Lipids, Inc) was a gift from Dr. Yan Xu, Cleveland Clinic Foundation. Colloidal gold, about 20 nm in diameter, was prepared by reducing Gold (III) chloride (0.01% aqueous solution of $HAuCl_4{\cdot}4H_2O$) (100 mL) with 1% aqueous solution of tri-sodium citrate (4 mL).[8] Immunogen was prepared by mixing LPA (1.1 mg) and the colloidal gold solution (60 mL) and stabilized by PEG (120 mg). The antisera were obtained from four immuned rabbits and purified with caprylic acid-ammonium sulfate.[9,10]

Dot immunobinding assay

A nitrocellulose (NC) membrane, with the purified antiserum dot, was soaked in gold-labelled antigen solution and let dry. The color of the dot was observed under visible light. To avoid the inference of colloidal gold antibody, the antiserum was re-purified with colloidal gold.

Dot immunogold filtration assay

A nitrocellulose (NC)-acetyl cellulose (AC) membrane was dropped with diluent re-purified antiserum. 0.01 M pH 7.4 PBS(control), 0.5 μg/mL LPA and 1 μg/mL LPA were mixed with equal volume of gold-labelled antigen solution and dropped onto the same dot, where the antiserum was immobilized, respectively. The color of the dot could also be observed under visible light.

Abbreviations: LPA, lysophosphatidic acid; PEG, polyethylene glycol; PBS, phosphate buffer saline solution; PC, phosphocholine; PA, phosphatidic acid.

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Results and Discussions

Usage of colloidal gold

Because of the low antigenicity of LPA that is a low-molecular-weight phospholipid, it has always been difficult to induce an immune response from animals and produce antibody against LPA. Usually, antibody can be induced by a conjugant of hapten and heterogenous bio-macromolecules or proteins. In this case, however, the antibody induced by conjugating the hydroxyl at sn-2 position or phosphate at sn-3 position of LPA with a protein may have high cross-reactivity with phosphatidic acid (PA) or lysophospholipid. On the other hand, the ω-alkyl at sn-1 position cannot couple with protein except for introducing an additional active functional group using chemical synthesis.

To avoid any possible side reaction in organic synthesis and production of other correlative antibodies, a simple preparation method of immunogen is attaching hapten to a certain kind of high-molecular-weight polymers and grains, such as carboxymethyl cellulose (CMC), polyvinyl pyrrolidone (PVP), active carbon and even colloidal gold,[6] in some kind of non-covalent interaction.

In this study, colloidal gold served a dual purpose. In order to prepare the immunogen, it was used as a high-molecular-weight carrier to attach LPA hapten. It also served as a direct indicator to the reactions between the antigen and antibody, thus simplifying the whole detection process.

Production and identification of antibody

The reaction between antigen and antibody occurred when the NC membrane, with the antiserum dot, was soaked in the gold-labelled antigen solution. The result was positive if a red dot showed on the membrane. According to Table 1, the antisera from all four rabbits, diluted 10 times and 100 times, had combined with gold-labelled antigen. When diluted 1000 times, only the antisera from rabbit **1** and rabbit **4** still combined with the gold-labelled antigen. This result proves that the LPA antibody was induced from all four immuned rabbits and the antisera from rabbits **1** and **4** had higher titers than those from rabbit **2** and **3**.

Specificity of antibody to LPA

Since the immunogen consisted of LPA, colloidal gold and PEG, it was possible that besides the LPA antibody there were also colloidal gold antibody and PEG antibody in the antiserum. By re-purifying the antiserum with colloidal gold, the possible interference from colloidal gold antibody was eliminated. And, the PEG antibody was not a factor, since the gold-labelled antigen that was used to identify the LPA antibody, was PEG free.

The results in Table 2 have shown that the red color appeared when purified or re-purified antiserum combined with gold-labelled antigen and no color when combined with gold-BSA, a control sample. It means that the antibody had no cross-reactivity with either colloidal gold or BSA.

Table 1. Identification of antibody in dot immunobinding assay

Rabbit	Reciprocal dilution of antisera		
	10	100	1000
1	++[a]	++	+
2	+	+	−[b]
3	+	+	−
4	++	++	+

[a] + = Red dot.
[b] − = No color.

Table 2. Specific reactivity of antibody with gold-labelled antigen

Antibody	Reciprocal dilution	Antigen	Display
Purified antiserum[a]	100	Gold-LPA-BSA[b]	+[d]
Re-purified antiserum[a]	100	Gold-LPA-BSA[b]	+
Re-purified antiserum[a]	100	Gold-BSA[c]	−[e]

[a] Antiserum from rabbit **4**.
[b] Gold-labelled antigen.
[c] Control.
[d] + = Red dot.
[e] − = No color.

Some compounds, with structures related to LPA, were used to test the cross-reactivity with the antibody using dot immunogold filtration assay. As it is mentioned later, 500 ng/mL of free LPA could inhibit the combination of the gold-labelled antigen with immobilized antibody, thus the red color of the dot disappeared. If any other compound has reactivity with LPA antibody, it is also able to inhibit the combination of gold-labelled antigen with antibody, and then the red color should disappear.

It is shown in Table 3 that when 50 μg/mL (hundred-fold of 500 ng/mL) of palmitic acid, glycero monostearate, dipalmitoyl phosphatidic acid, dipalmitoyl phosphocholine (PC) and LPA (18:1) were tested respectively, red dots could be observed due to the incapability to inhibit the combination of LPA antibody and gold-labelled antigen(16:0). The results suggested no cross-reactions occurred between those related compounds and the LPA antibody, which was produced with an immunogen of LPA (16:0).

Detection of LPA using dot immunogold filtration assay

In dot immunogold filtration assay, gold-labelled or free LPA was condensed onto membrane by filtration and reacted rapidly with the immobilized antibody. Positive result showed up as red dot because of the red colloidal gold. The test was easy, fast, and additional indicator was not necessary. Using this method, the free LPA could be detected qualitatively.

According to the report of Y. Xu[6] et al., a cutoff value of 1.3 μM (around 500 ng/mL) was used to define elevated LPA levels of patients in comparison with the healthy in their tests. The detectability of 500 ng/mL of LPA will inspire us with hope on the oncoming of practical diagnosis of ovarian cancer.

Table 3. Cross-reactivity of antibody with structurally related compounds

Antibody[a]	LPA in Ag[b] (μg)	Related compound		Display
2μL	0.25	Palmitic acid	50μg/mL	+[d]
2μL	0.25	Glycero monostearate	50μg/mL	+
2μL	0.25	PA, dipalmitoyl	50μg/mL	+
2μL	0.25	PC, dipalmitoyl	50μg/mL	+
2μL	0.25	LPA(18:1)	50μg/mL	+
2μL	0.25	LPA(16:0)	500ng/mL	–[e]
2μL	0.25	0[c]	0	+

[a]Re-purified antiserum from rabbit **4**, diluted 1:100 in PBS.
[b]Gold-labelled antigen.
[c]PBS (control).
[d]+ = Red dot.
[e]– = No color.

Table 4. Detection of free LPA in dot immunogold filtration assay

Antiserum[a]	LPA in Ag[b] (μg)	Free LPA		
		0[c]	250ng/mL	500ng/mL
2μL	1.00	++[d]	++	+
2μL	0.50	+	+	–
2μL	0.25	+	+	–
2μL	0.13	+	+	–
2μL	0.06	–[e]	–	–

[a]Re-purified antiserum from rabbit **4**, diluted 1:100 in PBS.
[b]Gold-labelled antigen.
[c]PBS (control).
[d]+ = Red dot.
[e]– = No color.

It is shown in Table 4 that red dot could be observed when LPA antibody combined with gold-labelled antigen. When 250 ng/mL of LPA (final concentration) was added, the free antigen level was too low to inhibit the combination of the gold-labelled antigen with immobilized antibody. Therefore, the color was still red (Column 4). When 500 ng/mL of LPA (final concentration) was added, the free antigen level was high enough to prevent the gold-labelled antigen from combining with the antibody. As a result, the color of dot became lighter or disappeared (Column 5). The data indicate that the LPA level higher than 500 ng/mL could be detected and the LPA level lower than 250 ng/mL could not be. In other words, when the amount of LPA in the gold-labelled antigen is between 0.13 μg and 0.05 μg, the free LPA higher than 500 ng/mL can be detected using the dot immunogold filtration assay.

Conclusions

The polyclonal antibody against LPA was successfully produced with an immunogen that was prepared by using colloidal gold as a hapten carrier to attach LPA in a non-covalent interaction. Using the dot immunogold filtration assay, the antibody could be used to qualitatively detect LPA levels higher than 500 ng/mL without cross-reactivity with the carrier, correlated protein and some compounds with structures related to LPA. Such antibody may be an important tool to the early diagnosis of ovarian cancer. It may also be useful for further studies of the biological functions of LPA in the differentiation and propagation of ovarian cancer cells.

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