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Original Article

Preparation of a nitric oxide imaging agent from gelatin derivative micelles

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ABSTRACT

Introduction: Nitric oxide (NO) is an intracellular and intercellular messenger that plays an important role in cellular events in physiological and pathophysiological processes. NO is one of the inflammation markers and macrophages of an inflammatory cell produce a large amount of NO compared with other cells. Non-invasive detection system of NO is highly required to realize an early therapeutic treatment considering the process of pathophysiological changes. The objective of this study is to develop an imaging agent of nitric oxide (NO).

Methods: A water-insoluble DAR-4M of fluorescent dye for NO was solubilized in water through the micelle formation with gelatin grafted with L-α-phosphatidylethanolamine distearoyl (DAR-4M micelles). Physicochemical and biological properties of DAR-4M micelles were investigated by using cultured cells and animals.

Results: The DAR-4M micelles responded to NO secreted from a NO donors, in contrast to the same concentration of free DAR-4M. When RAW264.7 of a macrophage cell line was stimulated by lipopolysaccharide (LPS) to allow them to generate NO, the DAR-4M micelles could detect NO of the cells to a significant great extent compared with free DAR-4M. After the intravenous injection of DAR-4M micelles or free DAR-4M to a mouse model of aristolochic acid (AA) induced acute interstitial nephritis, the DAR-4M micelles enhanced the fluorescence intensity from the kidneys to a significant great extent compared with the free DAR-4M injection. In case of DAR-4M micelles injection into normal mice, such an enhanced kidney fluorescence was not observed. A body distribution experiment demonstrated that the kidney accumulation of DAR-4M micelles was not modified by the AA-induced inflammation. After the AA injection, the number of CD11b-positive cells increased with time, indicating the increased number of inflammatory macrophages.

Conclusion: DAR-4M micelles are effective in imaging NO generated from macrophages accompanied with inflammation.

1. Introduction

Inflammation is one of the biological responses to various exogenous and endogenous stimuli which cause the cellular and tissue injuries. Recently, it has been demonstrated that the inflammation and regeneration are strongly related to each other [1]. For example, the regeneration is not occurred without inflammation. The extent and duration of inflammation reaction affect the subsequent regeneration process. In this context, it is no doubt that the inflammation imaging contributes to the development of efficient regenerative therapy. Macrophages are one of immune cells which play a critical role in inflammation responses by modulating the biological functions [2]. Therefore, it is important to develop the materials and technologies to control and visualize the biological functions of macrophages.

Nitric oxide (NO) is an intracellular and intercellular messenger that plays an important role in cellular events in physiological and pathophysiological processes [3–6]. NO acts as a vascular relaxing agent, a neurotransmitter, and an inhibitor of platelet aggregation. In addition, it is recognized that NO is generated during immune and inflammatory responses [7]. It functions in innate immunity as

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Abbreviations

NO	nitric oxide
nNOS	neuronal NOS
eNOS	endothelial NOS
DSPE	L-α-phosphatidylethanolamine distearoyl
DSPE-NHS	N-(Succinimidyloxy-glutaryl)-DSPE
LPS	lipopolysaccharide
SNP	nitroprusside
EtOH	ethanol
aDMSO	anhydrous dimethyl sulfoxide
DDW	double-distilled water
DMEM	Dulbecco's Modified Eagle Medium
FCS	fetal calf serum
WST-8	2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl) -2H- etrazolium
AA	aristolochic acid
HBSS	Hank's balanced salt solution
7-AAD	7-Amino-Actinomycin D
CMC	critical micellar concentration

a toxic agent towards infectious organisms, while it can induce or regulate death and function of host immune cells. Nitric oxide synthase (NOS) of enzyme to generate NO has been extensively investigated [8–10]. NO is generally biosynthesized by three isoforms of mammalian NOS [11]. Neuronal NOS (nNOS or NOS I) and endothelial NOS (eNOS or NOS III) are constitutively expressed in neuronal and endothelial cells, respectively. Both are also referred to as cNOS. Inducible NOS (iNOS or NOS II) is expressed in cells involved in inflammation, such as macrophages and microglias when stimulated by cytokines and/or endotoxins. Generally, the level of NO produced by cNOS in stimulated endothelial and neuronal cells is much lower (nanomolar [nM] range) than that generated by iNOS in macrophages (micromolar [μM] range). It is recognized that an excessive production of NO is believed to be responsible for various pathophysiologies, but very low levels of NO are needed to maintain normal physiological conditions. The NO production is closely associated with the expression of NOS [12].

Among several NO detecting dyes developed [13–17], DAR-4M is one of fluorescence dyes and has been used as the NO detecting agents in both in vitro and in vivo systems. Since the DAR-4M emits the fluorescence only in the presence of NO, the NO detection with a high signal-to-noise ratio is realized [13]. However, there are issues to be improved for the in vivo use. The DAR-4M is a small and water-insoluble molecule which is easily diffused and disappear in the body. Thus, it is practically difficult to maintain high concentrations at the site of NO to be detected. Several researches have been reported to improve the imaging efficiency of dyes by making use of materials sciences [18–21].

Gelatin is a biodegradable material and has been extensively used for food, pharmaceutical, and medical purposes. The biosafety has been proven through their long practical applications. Gelatin is a denatured form of collagen which is the most abundant component of extracellular matrix in the body tissue. The material itself and the product degraded are both biocompatible. It is also reported that the gelatin pre-coating of microspheres augments the phagocytosis of macrophages [22]. Hydrophobic derivatives of gelatins are effective in water-solubilization of water-insoluble and low-molecular weights drugs and enhancing their biological activity [23].

This study is undertaken to design the water-soluble micelles of DAR-4M for improved NO detection. The DAR-4M was solubilized in water by the micelle formation with a derivative of gelatin grafted with L-α-phosphatidylethanolamine distearoyl (DSPE) (DAR-4M micelles). The ability of DAR-4M micelles to image NO was evaluated by the in vitro cell culture with macrophages and an inflammation model at mice in vivo. We compare the NO imaging ability with that of free DAR-4M.

2. Materials and methods

2.1. Materials

Gelatin with an isoelectric point (pI) of 5.0 (weight-average molecular weight (Mw) = 10,000), prepared via an alkaline process of bovine bone (pI5 gelatin) was kindly supplied from Nitta Gelatin Co., Osaka, Japan. N-(Succinimidyloxy-glutaryl)-L-α-phosphatidylethanolamine, distearoyl (DSPE-NHS) was purchased from NOF Co., Tokyo, Japan. Diaminorhodamine-4M (DAR-4M) was purchased from Sekisui Medical Co., Ltd., Tokyo, Japan. Collagenase D was purchased from Roche diagnosis Inc., and used in the in vivo study because of its lower cytotoxicity compared with collagenase L. Lipopolysaccharide (LPS), nitroprusside (SNP) as a NO donor, and other chemicals were purchased from Wako Pure Chemical industries, Ltd., Osaka, Japan.

2.2. Synthesis of gelatin grafted with DSPE

PI5 gelatin with Mw of 10,000 (1.0 g) was dissolved in anhydrous dimethyl sulfoxide (aDMSO) at room temperature. DSPE-NHS (1.0 g) was dissolved in aDMSO at room temperature. Next, the gelatin solution in aDMSO was slowly added to the DSPE-NHS solution in aDMSO to give the DSPE/gelatin molar ratios of 1, 3, 5, and 10, and then stirred overnight at room temperature to allow DSPE to graft to gelatin. The resulting solution was dialyzed by double-distilled water (DDW) similarly for 72 h at room temperature, and freeze-dried to obtain DSPE-grafted gelatin samples (DSPE-g-gelatin). The extent of DSPE grafted to gelatin and the critical micellar concentration (CMC) of DSPE-g-gelatin were determined by the conventional molybdenum blue method to measure the phosphorus amount [24] and the fluorescence quenching of pyrene in DSPE-g-gelatin [25], respectively.

2.3. Water-solubilization of DAR-4M by DSPE-g-gelatin

DAR-4M solution (2.1 mg/ml) in aDMSO (4.0 μl) was added to 5 ml of DSPE-g-gelatin aDMSO solution (4.0 mg/ml), followed by 1 h stirring at room temperature. Then, the mixed solution was dialyzed with Spectra/Por®Dialysis Membrane MWCO: 3500 (Spectrum Laboratories, Inc, CA) against DDW for 72 h at room temperature. The dialysate obtained was centrifuged (8000 rpm, 10 min, 25 °C) to exclude the water-insoluble fraction, and freeze-dried to obtain the DAR-4M water-solubilized by DSPE-g-gelatin micelles (DAR-4M–micelles). The size of DAR-4M micelles was measured by a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, UK). The amount of DAR-4M water-solubilized in each micelle was determined by measuring the absorbance of DAR-4M at the wavelength of 315 nm after dissolving each micelle in aDMSO.

2.4. Evaluation of the DAR-4M micelles response to NO donors

The response of DAR-4M micelles to NO donors was evaluated according to the conventional fluorescence measurement [13]. Briefly, DAR-4M micelles at the DAR-4M concentration of 10 μM (3.14 mg/ml) were added into 10 mM phosphate-buffered saline (PBS, pH 7.4) or SNP/PBS solution in each well of 96-well multi-well black plate (Corning Inc., Corning, NY). The fluorescent intensity of