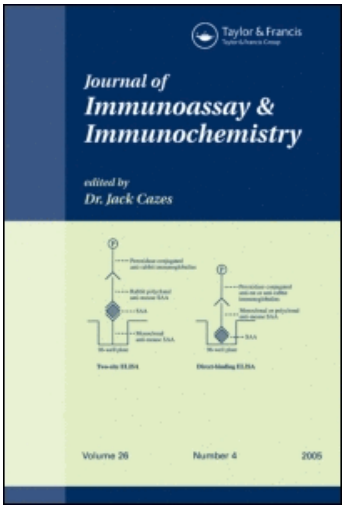

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CHARACTERIZATION OF MONOCLONAL ANTIBODY REACTIVE WITH 10-HYDROXY-12(Z)-OCTADECENOIC ACID (10-OHODA) AND ITS DEMONSTRATION IN CULTURED HUMAN MACROPHAGES

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ABSTRACT

10-Hydroxy-12(Z)-octadecenoic acid (10-OHODA) has an inhibitory effect on the tension of papillary muscles in isolated guinea-pig hearts. To establish an immunoassay for 10-OHODA a mouse monoclonal antibody (MoAb), YM-1, was produced. In order to evaluate the ability of this MoAb to recognize various 10-OHODA analogs including leukotoxin (9,10-epoxy-12-octadecenoic acid, LTx), a sensitive enzyme-linked immunosorbent assay (ELISA) was developed using the avidin-biotin complex (ABC). The detection limit for 10-OHODA was as low as 0.5 ng in this system. In order to demonstrate the presence of 10-OHODA in living cells, macrophages derived from the human leukemia cell line THP-1 by adding 160nM phorbol 12-myristate 13-acetate (PMA) were exposed to 95% O_2, and 5% CO_2 for 24 h. 10-OHODA and other fatty acids were extracted from the exposed macrophages with diethylether after phospholipase A_2 treatment. The 10-OHODA content was determined using the new ELISA, and 18.5 ng 10-OHODA was detected in the macrophages exposed to the high oxygen concentration (1×10^6cells).
(KEY WORDS: 10-Hydroxy-12(Z)-octadecenoic acid, Monoclonal antibody, Leukotoxin, Enzyme-linked immunosorbent assay, Macrophage, superoxide.)

INTRODUCTION

10-Hydroxy-12-octadecenoic acid (10-OHODA, Fig.1) is an unsaturated fatty acid mainly known as one of the components of human adipocere in the field of forensic medicine (1). Ozawa et al. also demonstrated the existence of a small amount of 10-OHODA in human polymorphonuclear leukocytes (2). This compound has been considered to have an important effect on the circulatory system (3) and we have previously described its suppressant effect on papillary muscle contractile force in guinea pigs (4). The dynamics of 10-OHODA in living beings and its biological activity are currently attracting much attention in the pathophysiological field.

In order to investigate the dynamics of 10-OHODA, it is necessary to produce an antibody specific to 10-OHODA and to develop a highly sensitive and convenient assay system, such as an immunoassay. In this paper, we report the development of a sensitive enzyme-linked immunosorbent assay (ELISA) system for 10-OHODA using a mouse monoclonal antibody which recognizes 10-OHODA with high specificity. We also demonstrate the presence of 10-OHODA in cultured human macrophages.

MATERIALS AND METHODS

Chemicals

Silica gel 60HF was supplied by Merck, Darmstadt, Germany. [^{14}C] Linoleic acid (1.5 kBq, specific activity 1.0 Ci/mmol) was purchased from New England Nuclear Co.,USA. Phorbol 12-myristate 13-acetate (PMA), linoleic acid and oleic

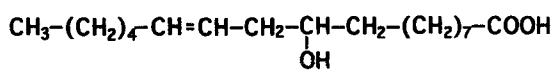

Fig.1: Structure of 10-OHODA (10-Hydroxy-12-octadecenoic acid)

acid were supplied by Sigma Chemical Co., Mo., U.S.A., and other fatty acids used for cross-reactivity studies were synthesized organically or biochemically (5-7). Organic solvents for HPLC and other reagents were purchased from Wako Pure Chemical Ltd., Osaka, Japan. Biotinylated horse anti-mouse IgG(H+L) and a standard Vectastain ABC (biotinylated alkaline phosphatase kit) were purchased from Vector Laboratories, U.S.A. All other reagents were obtained from Nakarai Chemical Co., Kyoto, Japan.

Preparation of immunogen

10-OHODA was synthesized biologically from linoleic acid by *Lactobacillus plantarum* (8) . The resulting mixture of fatty acids was separated by thin-layer chromatography (TLC) on plates coated with silica gel 60HF using a solvent system of petroleum ether/diethyl ether/acetic acid (70:30:1, by vol.). The isolated 10-OHODA spot was scraped off and eluted from the silica gel with diethyl ether. The eluted fatty acid was repeatedly purified by TLC until the HPLC analysis (9) indicated 10-OHODA was more than 99% pure.

The synthesized 10-OHODA was conjugated to bovine serum albumin (BSA) and to keyhole limpet hemocyanin (KLH) according to the method of Erlanger et al (10). For estimation of the amounts of 10-OHODA coupled to BSA and KLH, the radioactivity of [^{14}C] linoleic acid in the dialysate was measured. The degree of con-

jugation was calculated to be 27 mol per one mol of BSA (M.W. 68,000), and 1,540 mol per one mol of KLH (average M.W.5,250,000).

Monoclonal antibody production

Six-week-old BALB/c mice were first immunized intraperitoneally with 250μg 10-OHODA-BSA in saline, emulsified 1:1 in complete Freund's adjuvant. Mice then received intraperitoneal injections of 250μg 10-OHODA-BSA in saline only, monthly for 6 months. Three days after the final injection, their spleens were removed, fused with the P3U1 myeloma cell line using PEG 1500, and grown under the conditions described by Köhler and Milstein (11). Hybridomas producing the antibody for 10-OHODA (YM-1) were screened by an ELISA. One hybridoma cell from a positive well was subcloned twice using a limiting dilution to ensure monoclonal origin. An isotype analysis of the antibody was performed by the double diffusion technique of Ouchterlony (12).

ELISA procedure

The ELISA procedure was basically carried out as described previously (13). 10-OHODA-KLH was used as a solid phase in this ELISA system. 10-OHODA and other lipids were first dissolved in methanol, and each working solution was adjusted to contain 5% methanol with 10mM phosphate-buffered saline (PBS, pH 7.4).

Cell treatment

A human monocytic leukemia cell line, THP-1 (14,15), was maintained in suspension culture in RPMI 1640 tissue culture medium supplemented with 10% fetal calf serum and kanamycin (60μg/ml), and was incubated for 24 h. PMA was solubi-

lized in DMSO at a concentration of 1.6μM and added to the THP-1 cell cultures at various concentrations.

Sample treatment

After a given period of culture, the tissue culture medium was divided between two bottles. One bottle was exposed to oxygen-rich conditions (95% air, 5%CO_2) for 24 h. The other was continuously exposed to ambient air for 24 h. The room temperature was 20℃-25℃. Suspension cultures of treated THP-1 cells were centrifuged, then the cells were resuspended in 5 ml 10 mM PBS (pH 7.4) containing 2 mM $CaCl_2$ and 100 units PLA_2(phospholipase A_2), and incubated for 2 h at 37℃. Fatty acids were extracted twice with diethyl ether, after the pH of the mixture had been adjusted to 2-3 by adding a 2.5% phosphate solution. A concentration of 10-OHODA and leukotoxin (9,10-epoxy-12-octadecenoic acid, LTx) was measured by ELISA using YM-1 and an anti-LTx-monoclonal antibody (16). In addition, the cells were checked to determine whether any which had changed from the THP-1 type had the functional characteristics of mature macrophages. Phagocytic activity and adherence of macrophages were measured by the fluorometric assay method (17) and the NBT method (18), respectively.

RESULT AND DISCUSSION

After cell fusion, hybridomas were observed in more than 90% of the wells in the microculture plate. Finally, one clone (YM-1) was established. The immunoglobulin subclass of this clone was IgG_{2a}. The ELISA performed satisfactorily when a 1:400 dilution of the culture fluid was used as the monoclonal antibody. Fig. 2

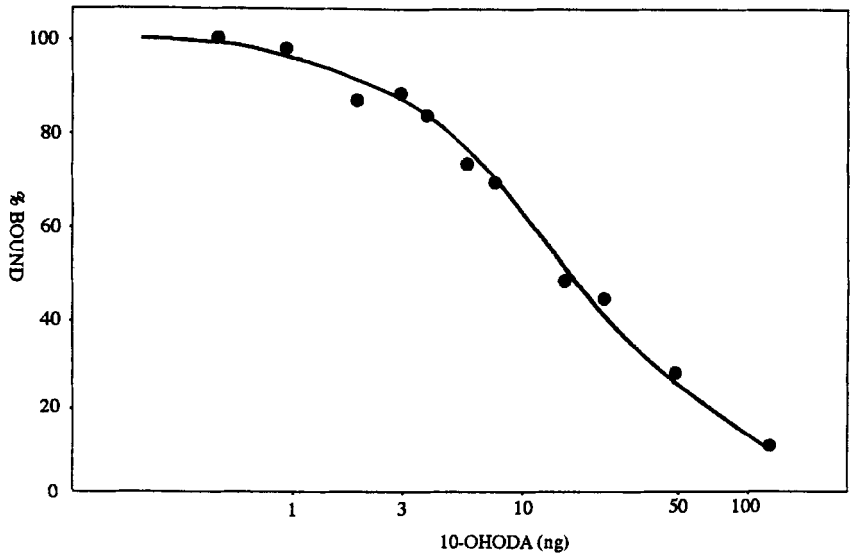

Fig.2: Inhibition of binding of the monoclonal antibody to 10-OHODA-KLH in the ELISA by 10-OHODA

shows the behavior of the monoclonal antibody in the ELISA. The decrease in color development was almost linear over the range 6 to 30 ng 10-OHODA. In this ELISA system, the amount of 10-OHODA causing 50 % inhibition was 16.5 ng. The intra-assay coefficient of variation for 10 duplicate determinations of each sample was 7.4%, and its inter-assay coefficient of variation for 10 duplicate determinations was 9.7%. The specificity of YM-1 was evaluated by cross-reactivity studies with 10-OHODA and 6 other fatty acid analogs. As shown in Table 1, 10-hydroxy stearic acid (10-Hydroxy-$C_{18:0}$) showed 10.7% cross-reactivity with this antibody. The other analogs, including LTx, exhibited little reactivity with YM-1. The concentrations of fatty acids in the cultured macrophages were determined by

ELISA using monoclonal antibodies; the levels of 10-OHODA and LTx in the macrophages exposed to 95% oxygen were 18.5 ng/10^6cells and 17.4 ng/10^6cells, respectively. On the other hand, the levels of 10-OHODA and LTx in the macrophages exposed to ambient air were 6.9 ng/10^6cells and 5.3 ng/10^6cells, respectively (Fig. 3). Table 1 shows YM-1 is very specific to 10-OHODA and that among the analogs of 10-OHODA, only 10-hydroxy stearic acid is recognized to any extent by this antibody, suggesting that YM-1 recognizes not only the 10-hydroxy group but also the 12:13-double bond. This ELISA system can detect concentrations of 10-OHODA at least as low as 0.5 ng and would therefore be useful in detecting traces of 10-OHODA in samples without the need for radioisotopic materials. 10-OHODA is a hydroxy fatty acid derived from linoleic acid, and the presence of a very small amount of 10-OHODA has been reported in polymorpho-nuclear cells (2). However the metabolic pathway by which is produced is still unclear. We previously reported that 10-OHODA produced from linoleic acid by *Lactobacillus plantarum* has an inhibitory effect on the tension of papillary muscles in isolated guinea-pig hearts, as dose LTx (4). LTx has also been found to play an important role in the pathophysiology of diseases such as cardiac infarction, inflammatory diseases and trauma (5,6). We previously reported that LTx was produced in both alveolar macrophages and neutrophils in the lungs of rats under hyperoxic conditions (19). Cross et al. (20) reported that free radicals such as superoxide and/or the hydroxy radical lead to cell damage via lipid peroxidation of many unsaturated fatty acids in cell membranes. LTx produced via a linoleate cascade appears to be closely

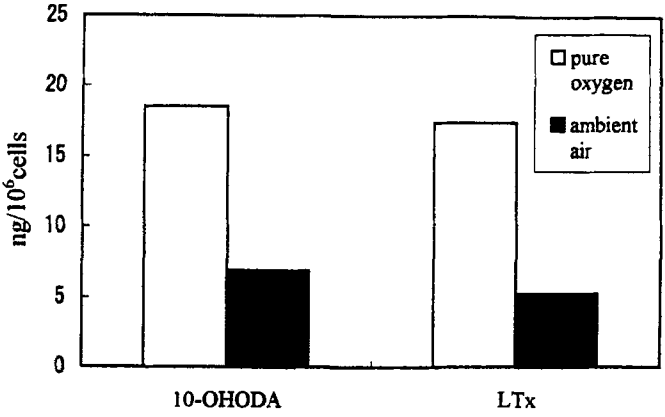

Fig.3: Quantitative comparisons of 10-OHODA and LTx in macrophages between 2 type culture conditions measured by ELISA (ng/10^6 cells)

Table 1. Specificity of the anti-10-OHODA monoclonal antibody

Analog	Structure	Crossreactivity (%)
10-Hydroxy-$C_{18:1}$	$CH_3-(CH_2)_4-CH=CH-CH_2-CH(OH)-CH_2-(CH_2)_7-COOH$	100
10-Hydroxy-$C_{18:2}$	$-CH-CH_2-CH_2-CH(OH)-CH_2-$	10.7
LTx	$-CH=CH-CH_2-CH(O)-CH-$	0.06
LTx'	$-CH-CH(O)-CH_2-CH=CH-$	0.04
9:10-Epoxy-$C_{18:0}$	$-CH_2-CH_2-CH_2-CH(O)-CH-$	0.04
Linoleic acid	$-CH=CH-CH_2-CH=CH-$	0.04
Oleic acid	$-CH_2-CH_2-CH_2-CH=CH-$	0.03

involved in cell damage and/or remodeling (21). We found small amounts of both LTx and 10-OHODA in the cultured human macrophages exposed to oxygen or ambient air in the present study. Production of both compounds was greater when the cells were exposed to pure oxygen than with ambient air (Fig. 3), strongly suggesting that the production of these substances in macrophages is attributable to free radical species (22-24) . We suspect that 10-OHODA, in combination with LTx, is involved in biological reactions with in macrophages, acting as some kind of chemical mediator. In this study we demonstrated the production of an anti-10-OHODA monoclonal antibody and the development of a sensitive and reliable ELISA for 10-OHODA using this antibody. We also demonstrated the presence of 10-OHODA in cultured human macrophages

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