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Influence of the culture medium on the production of nitric oxide and expression of inducible nitric oxide synthase by activated macrophages *in vitro*

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ABSTRACT

Macrophages play an important role in immune and inflammatory responses, and have been extensively studied *in vitro* using culture media such as RPMI1640 medium, Dulbecco's modified Eagle medium (DMEM), and Ham's F-12 medium (F-12). We found that the activation phenotypes of a murine macrophage-like cell line, J774.1/JA-4, were obviously different in two distinct culture media (F-12 and DMEM), both of which were supplemented with 10% of the same fetal bovine serum (FBS). Among these phenotypes, nitric oxide (NO) production as well as inducible NO synthase (iNOS) expression, induced by lipopolysaccharide (LPS) and interferon-γ (IFN-γ), were remarkably different. iNOS expression was higher in the macrophages cultured in DMEM than in F-12 for 20 h, while no significant differences were shown in NO production between in F-12 and DMEM. It might be the reason why DMEM have reduced NO production by the induced iNOS. Besides, O_2^--generating activity, and production of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in the activated macrophages were also different between the cultures in F-12 and DMEM. These results suggest that F-12 and DMEM contain certain components responsible for modification of macrophage activation processes and/or macrophage functions. Our present results provide evidence that the choice of culture medium is important in the study and analysis of macrophage activation.

1. Introduction

Macrophages play important roles in innate immune responses to pathogens, tumor cells and apoptotic cells of the host [1–4]. Macrophages also change their properties through activation processes [5,6]. Activated macrophages have the property to produce reactive oxygen species (O_2^- and H_2O_2), nitric oxide (NO), and pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [7–10]. Because these molecules act on pathogens or immune cells directly, our body can maintain homeostasis [4]. Many of these findings were obtained by culturing of primary macrophages and macrophage-like cell lines in various culture media *in vitro*. According to Hou et al. [11], different effects on cell proliferation and differentiation were observed in periodontal ligament cells by using different types of culture media. Similarly, dental pulp-derived cells [12], periosteum-derived cells [13], and others exhibited different phenotypes as to cell functions with different culture media [14,15]. However, no results have been reported concerning the effects of different culture media on macrophage activation.

Various culture media, containing amino acids, vitamins, inorganic salts, and trace elements, are used widely *in vitro* by many researchers. Furthermore, serum (e.g., FBS and FCS), which contains albumin, cell growth factors, hormones, protease inhibitors, and so on, is added to facilitate the growth of or to protect cells. In a study involving macrophages, the endotoxin content of FBS also requires careful attention [16]. Compared with the influence of FBS on a cell culture, which is well known by many scientists, that of culture media is relatively unknown as to cell function except for cell growth or differentiation. Therefore many studies on macrophages involving various culture media might have resulted in different results among laboratories. We need to pay much attention to the influence of the culture medium in a variety of cell culture experiments.

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In this study, we present a series of novel evidence that a murine macrophage-like cell line, J774.1/JA-4, expresses different activated macrophage phenotypes induced by lipopolysaccharide (LPS) and/or interferon-γ (IFN-γ) on incubation in either Ham's F-12 medium (F-12) or Dulbecco's modified Eagle medium (DMEM). Production of NO and some cytokines was increased more in DMEM during macrophage activation than in F-12. We also examined the precise mechanisms underlying the induction and expression of inducible NO synthase (iNOS) and its activity.

2. Materials and methods

2.1. Materials

F-12, DMEM, and FBS were purchased from Thermo Fisher Scientific Inc (Waltham, MA, U.S.A). Recombinant murine IFN-γ was a generous gift from TORAY (Tokyo, Japan). Penicillin and streptomycin solution was purchased from Nacalai Tesque (Kyoto, Japan), and *Escherichia coli* 055:B5 LPS, chromatographically purified, phorbol myristate acetate (PMA), cytochrome *c* from horse heart, and superoxide dismutase (SOD) from bovine liver ($\geq$ 1500 units/mg protein) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other reagents and chemicals were of the purest commercial grade available.

2.2. Cell culture

Culturing of the JA-4 cell line, an LPS-sensitive subline of a murine macrophage-like cell line, J774.1, was performed as described previously [8]. In brief, cells were maintained and cultured in 10 mL of F-12 supplemented with 10% heat-inactivated FBS, 50 units/mL of penicillin, and 50 μg/mL of streptomycin in a 100 mm plastic dish (Falcon #351029; Corning Life Science, NY, U.S.A.) at 37 °C in a CO_2 incubator (5% CO_2-95% humidified air). The cells were passed every 1–3 days and were maintained until 25th passage without any significant cell morphological change or biological response.

2.3. Measurement of NO, TNF-α, and IL-1β production

Cells were seeded at 2×10^6 cells/4 mL/dish of F-12 onto 60 mm culture dishes (#430166; Corning Life Science), and then incubated at 37 °C for 2–4 h. The medium was replaced with 4 mL of fresh F-12 or DMEM medium containing LPS (100 ng/mL) and/or IFN-γ (10 units/mL), and then the cells were incubated at 37 °C for various durations (0–20 h). For measurement of NO, TNF-α, and IL-1β production, culture supernatants were collected and then centrifuged at 10,000 rpm (9,100*g*) at 4 °C for 1 min. Crude extracts were prepared from the cells for Western blot analysis. NO was measured as a stable form of nitrite ions (NO_2^-) by using Griess reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan). TNF-α and IL-1β production were analyzed by enzyme-linked immunosorbent assaying (ELISA) (R&D Systems, Minneapolis, U.S.A.).

2.4. Re-incubation of macrophages after activation, and measurement of endogenous NADPH

Cells were activated in F-12 or DMEM containing LPS (100 ng/mL) and IFN-γ (10 units/mL) at 37 °C for 20 h. The cells were then washed once with warm sterile phosphate-buffered saline (PBS), and re-incubated for various durations (0–6 h) after a change to fresh F-12 or DMEM medium containing none of the activating factors. At the different time points, culture supernatants were collected and then the cells of re-incubation at 4 h were then washed twice with ice-cold PBS, and endogenous NADPH was extracted and measured using a SensoLyte NADP/NADPH assay kit (AnaSpec Inc., California, U.S.A.) according to the manufacturer's instruction.

2.5. Measurement of O_2^--generating activity

O_2^--generating activity was examined as described before [8,17]. Cells were seeded at 1×10^5 cells/0.25 mL/well of F-12 onto 48-well plates (Costar #3548; Corning Life Science), and then incubated at 37 °C for 2–4 h. The medium was replaced with 0.25 mL of fresh F-12 or DMEM medium containing LPS (100 ng/mL) and/or IFN-γ (10 units/mL), and then the cells were incubated at 37 °C for 20 h. The cells were then washed twice with PBS, and 0.25 mL Hank's balanced salt solution containing cytochrome *c* (1 mg/mL) with/without SOD (10 μg/mL) were added. The reaction was initiated by the addition of PMA (1 μg/mL), and after incubation at 37 °C for 1.5 h, stopped by chilling of the plates on ice. The culture supernatants were collected and examined at the wavelength of 550 nm with a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan). The differences in A_{550} between the samples without and with SOD were determined, and the level of O_2^--generating activity was calculated as the reduction of cytochrome *c* on the basis of that 1 unit of optical density at 550 nm corresponds to 47.2 nmol of O_2^- [8]. To determine cell protein amounts, the cells were rinsed with PBS (−) twice, and the resultant monolayer cells were extracted and then used for estimation of cell protein amounts by the method of Lowry et al. [18].

2.6. Western blot analysis

As described previously, cells were stimulated with LPS and/or IFN-γ for various times. The cells were then chilled on ice and washed twice with ice-cold PBS, after which they were scraped into lysis buffer comprising 1% (v/v) Triton X-100, 2 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 10% glycerol, and 1% protease inhibitor cocktail (Nacalai Tesque) in 20 mM Tris–HCl, pH 7.4. The cell lysates were fragmented at 4 °C for 3 min (30 sec on/20 sec off, 60% duty) with a sonicator, ELESTEIN 05-01 (Elekon Science Co., Ltd., Chiba, Japan). Finally, the resultant solutions were centrifuged at 10,000 rpm at 4 °C for 1 min, and the resulting supernatants were used as cell extracts. 25 μg aliquots of the cell extracts were electrophoresed through a 5–20% gradient polyacrylamide gel (ATTO, Tokyo, Japan), and the proteins were transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Merck, Millipore, Billerica, U.S.A.). The membranes were blocked with 30 mg/mL milk casein (Megmilk Snow Brand, Tokyo, Japan) in a rinse buffer comprising 0.1% Triton X-100, 0.1 mM EDTA, and 0.8% NaCl in 10 mM Tris–HCl buffer, pH 7.4, and then incubated with mouse anti-iNOS/NOS Type II (BD Transduction Laboratories, New Jersey, U.S.A.), rabbit anti-TNF-α (Thermo Fisher Scientific Inc.), goat anti-IL-1β/IL-1F2 (R&D Systems), and mouse anti-β-actin (Sigma-Aldrich)-specific antibodies, respectively, at 4 °C overnight. The membranes were then reacted with a horseradish peroxidase-conjugated anti-rabbit, anti-goat, or anti-mouse immunoglobulin G (Cell Signaling Technology, Danvers, MA, U.S.A.)-specific antibody at room temperature for 1 h. The immune complexes on the membranes were detected by the addition of Pierce Western Blotting Substrate (Thermo Fisher Scientific Inc.). Chemiluminescence signals were detected using an LAS 3000 mini image analyzer (FUJIFILM, Tokyo, Japan), and the results were analyzed with Image J software (developed at the National Institutes of Health).