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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).

2.7. RNA isolation and quantitative reverse transcription-Polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells using Tripure Isolation Reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. RNA quality and concentration were assessed with a NanoDrop Light spectrophotometer (Thermo Fisher Scientific Inc). Total RNA was reverse-transcribed with ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). The forward and reverse primer sequences for each gene were as below, respectively: NOS2 (iNOS): 5′-CTTTGCCACGGACGAGAC-3′ and 5′-TCATTGTACTCTGAGGGCTGAC-3′; TNF-α: 5′-CTGTAGCCCACGTCGTAGC-3′ and 5′-TTGAGATCCATGCCGTTG-3′; IL-1β: 5′-AGTTGACGGACCCCAAAAG-3′ and 5′-AGCTGGATGCTCTCATCAGG-3′; and GAPDH: 5′-CAAGGAGTAAGAAACCCTGGACC-3′ and 5′-CGAGTTGGGATAGGGCCTCT-3′. Real-Time PCR was performed on an Applied Biosystems StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific Inc) using FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics GmbH), and the relative quantification (RQ) was performed using StepOne™ software V2.2.2 (Thermo Fisher Scientific Inc), based on the equation $RQ=2^{-\Delta\Delta Ct}$, where *Ct* is the threshold cycle to detect fluorescence. *Ct* data were normalized as to the internal control GAPDH mRNA.

2.8. Statistical analysis

The results are expressed as means ± S.D. or S.E. for at least three independent experiments. The difference between the two groups was analyzed using Student's *t*-test with the significance of difference being set at $p < 0.05$.

3. Results

3.1. The morphology of JA-4 cells activated in F-12 or DMEM

JA-4 cells exhibited the round-shape morphology in both media (Fig. 1). Moreover, when the cells were cultured in each medium containing IFN-γ alone, their morphologies were mostly round-shape without difference between both media. In contrast, when the cells were treated with LPS alone or LPS+IFN-γ, spindle-shaped cells appeared in both media frequently almost at the same rate (Fig. 1).

3.2. Effects of the culture medium on the iNOS expression and NO production in activated JA-4 cells

The expression of iNOS protein was induced significantly only by LPS+IFN-γ stimulation, the extent of the induction in DMEM being almost twice than in F-12 (Fig. 2A and B). Induction of iNOS protein in the course of macrophage activation was significantly higher in DMEM at every time point after 8 h than in F-12 (Fig. 2C and D), while the amounts of NO_2^- in these cell culture supernatants showed no significant differences between in F-12 and DMEM (Fig. 2E). To determine this difference was caused by either post-transcriptional or post-translational regulation, we analyzed the expression of iNOS mRNA in the course of macrophage activation. The expression of iNOS mRNA was also significantly higher in DMEM than in F-12 at 4 and 8 h after stimulation of macrophages with LPS+IFN-γ (Fig. 2F).

3.3. Effect of the culture medium on NO production by activated JA-4 cells during re-incubation in F-12 or DMEM

As shown in Fig. 2, differences in NO production were smaller than those in iNOS protein between the macrophages cultured in F-12 and DMEM after treatment with LPS+IFN-γ. These results suggest that DMEM might have reduced NO production by the induced iNOS in the activated macrophages. In order to examine this possibility, activated macrophages treated with LPS+IFN-γ at 37 °C for 20 h in either F-12 or DMEM were re-incubated further at 37 °C in the same or the other medium but without LPS or IFN-γ. NO production by the activated macrophages in either of the media became significantly higher during re-incubation in F-12 than in DMEM (Fig. 3A), while the medium change did not affect the amount of iNOS protein during the re-incubation (Fig. 3B). Moreover, NO production during the re-incubation increased linearly in either of the media up to 6 h (Fig. 3C). Then, we determined the amount of NADPH, an electron acceptor of iNOS, before and after the re-incubation in these media. Culturing in DMEM resulted in a significant reduction of about 20% of NADPH compared to that in F-12 during the re-incubation (Fig. 3D).

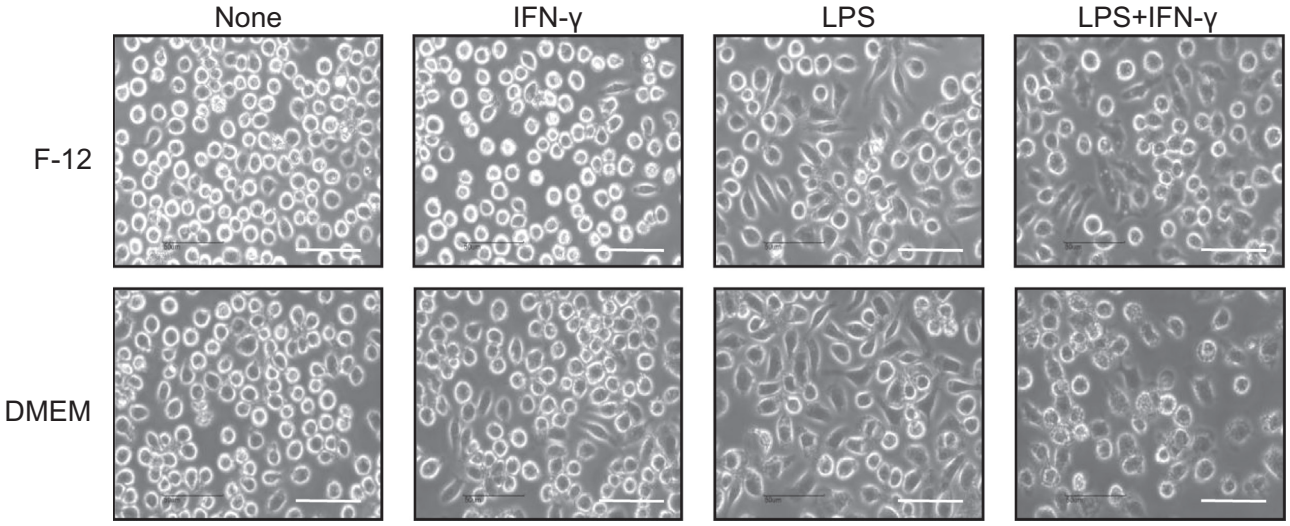

Fig. 1. Morphological changes of macrophages during incubation with LPS and/or IFN-γ in either F-12 or DMEM. J774.1/JA-4 cells were pre-incubated in F-12, and then the medium was replaced with either F-12 or DMEM containing IFN-γ (10 units/mL) and/or LPS (100 ng/mL), and the cells were then incubated at 37 °C for 20 h. Representative photographs from repeated experiments are shown. Magnification, × 200. Scale bar, 50 μm.

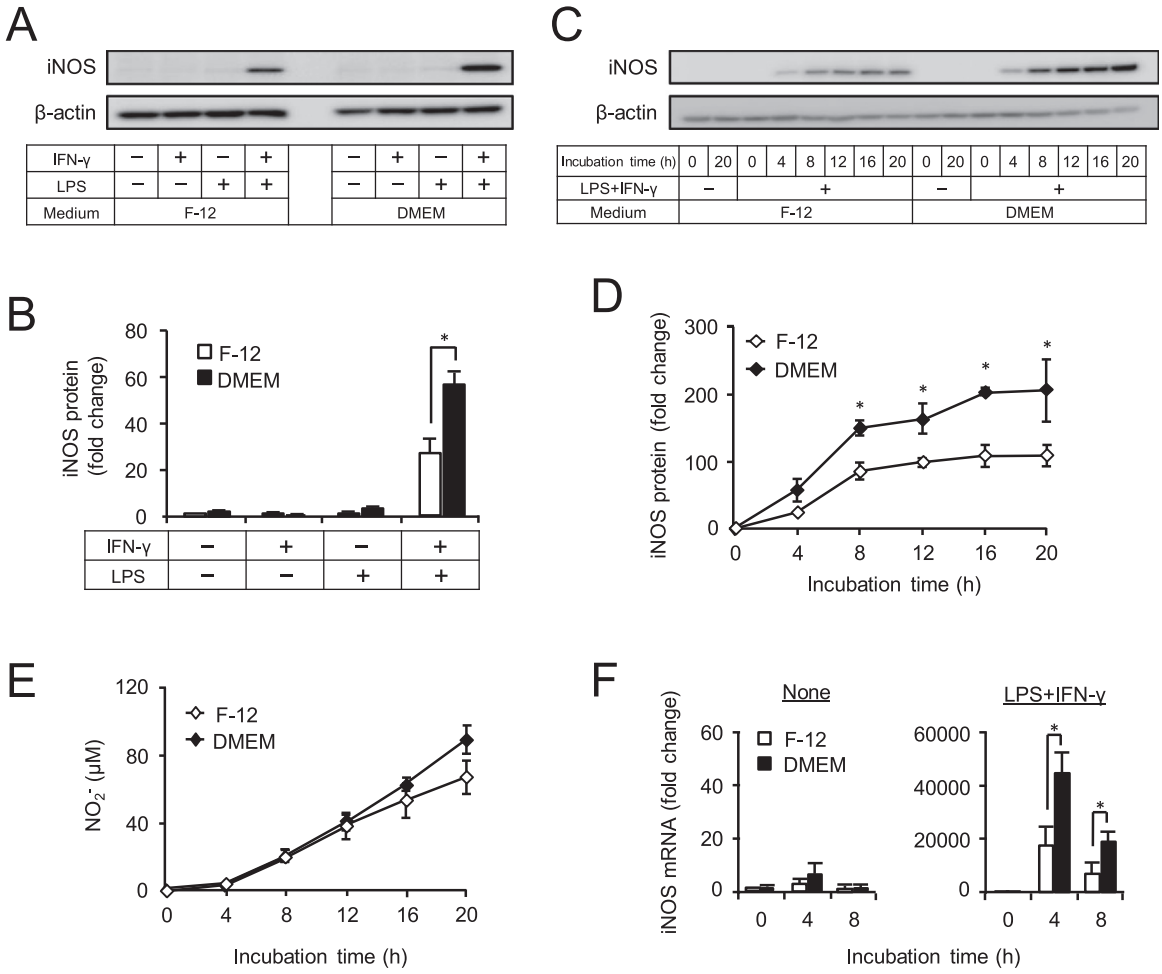

Fig. 2. Effects of culture medium on iNOS expression and NO production in the course of macrophage activation in F-12 or DMEM. (A) Western blot analysis of iNOS protein and β-actin using total protein extracts of JA-4 cells, cultured in F-12 or DMEM, in the presence or absence of IFN-γ (10 units/mL) and/or LPS (100 ng/mL). (B) Relative amount of iNOS protein. The results in (A) are quantitatively shown as values relative to those for the untreated cells in F-12 after normalization as to the amount of β-actin in each sample. Densitometry data are expressed as means ± S.D. for three independent experiments. $^{*}p < 0.05$ between indicated pairs of F-12 and DMEM. (C) Western blot analysis of iNOS protein in total protein extracts of JA-4 cells treated with LPS+IFN-γ in either F-12 or DMEM at 37 °C for the times indicated on the abscissa. (D) Time-course of iNOS protein induction in F-12 and DMEM. The results in (C) are quantitatively shown as values relative to those for the untreated cells in F-12 at 0 h after normalization as to the amount of β-actin in each sample. Densitometry data are expressed as means ± S.D. for three independent experiments. $^{*}p < 0.05$ between indicated pairs of F-12 and DMEM. (E) NO_2^- in the culture supernatants of macrophages after incubation with LPS+IFN-γ for the indicated times. NO_2^- was estimated by the Griess reagent assay. The results are shown as means ± S.D. for three independent experiments. (F) Expression of iNOS mRNA during macrophage activation in F-12 and DMEM. mRNA was extracted and quantitated by qRT-PCR, as described in the text. The results are relative to those for the untreated cells in F-12 at 0 h after normalization as to the internal control, GAPDH mRNA. The results are expressed as means ± S.D. for three independent experiments. $^{*}p < 0.05$ between indicated pairs of F-12 and DMEM.

3.4. Effects of the culture medium on O_2^--generating activity, and expression of TNF-α and IL-1β in activated JA-4 cells

The O_2^--generating activity in JA-4 cells treated with LPS and/or IFN-γ in either F-12 or DMEM was examined using the cytochrome *c* reduction assay. The activity was significantly lower in the cells cultured in DMEM containing LPS than those in F-12 (Fig. 4A). As for TNF-α and IL-1β expression in DMEM, both the precursor protein and mRNA of each cytokine in the activated macrophages were different (Fig. 4B). While expression of pro-TNF-α protein showed no significant differences between the cells treated with LPS and/or IFN-γ in these media (Fig. 4B and D), TNF-α production in DMEM was significantly higher than in F-12 at 16 h and later after treatment with LPS+IFN-γ (Fig. 4C, left). Besides, expression of pro-IL-1β protein was induced earlier in F-12 than DMEM at 12 h after treatment with LPS+IFN-γ, but the level of pro-IL-1β increased rapidly in DMEM after 12 h (Fig. 4C, right). Supporting these results, induction of pro-IL-1β protein was delayed in DMEM compared to that in F-12 (Fig. 4D), and the IL-1β mRNA level at 4 h after stimulation was significantly higher in F-12 than in DMEM (Fig. 4E).

4. Discussion

F-12 and DMEM are widely used for supporting the growth of various cells. However, there are some differences in their compositions; F-12, developed by Ham, contains various amino acids, vitamins, and trace elements, and DMEM, modified from Basal Medium Eagle, contains higher levels of amino acids, vitamins, calcium (6-fold), glucose (2.5-fold), and so on than F-12.

This study demonstrated that these culture media influence the activated macrophage phenotypes of the J774.1/JA-4 macrophage-like cell line. Among them, NO production and iNOS expression were the most remarkably influenced, because induction of iNOS mRNA and iNOS protein was higher in DMEM than in F-12, but NO production by the activated macrophages was less in DMEM than

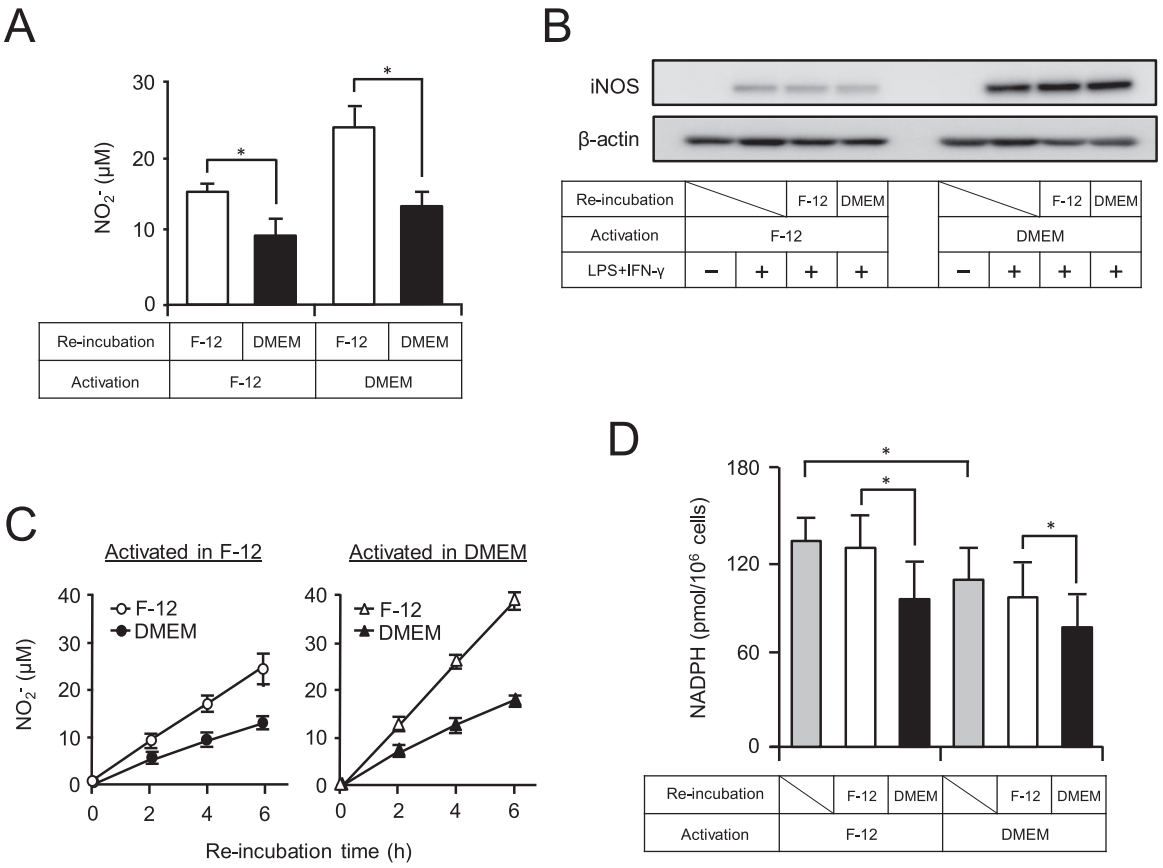

Fig. 3. Effect of culture medium on NO production by activated macrophages during re-incubation in F-12 or DMEM. Cells were pre-incubated in F-12 and then treated with LPS (100 ng/mL) and IFN-γ (10 units/mL) in F-12 or DMEM at 37 °C for 20 h. Then the cells were washed with warm-PBS, followed by re-incubation in either fresh F-12 or DMEM at 37 °C for 4 h. (A) NO_2^- in the culture supernatants of the macrophages during re-incubation. The results are expressed as means ± S.D. for three independent experiments. $^*p < 0.05$ between indicated pairs of F-12 and DMEM. (B) Western blot analysis of iNOS protein in total protein extracts of cells after activation for 20 h and subsequent re-incubation for 4 h. (C) Time-course of NO production during re-incubation of activated macrophages. NO_2^- in the culture supernatants of the re-incubated cells in either of the media is shown, as described in the text. (D) NADPH levels in activated macrophages during re-incubation in either F-12 or DMEM. NADPH was extracted from the re-incubated cells and analyzed with an NADP/NADPH assay kit, and the results are shown as means ± S.E. for seven independent experiments. $^*p < 0.05$ between indicated pairs of F-12 or DMEM.

in F-12, based on the amount of iNOS protein induced during macrophage activation and subsequent re-incubation of the activated macrophages (Fig. 3). Though iNOS is the key enzyme being, induced by macrophage activation with LPS+IFN-γ, NO production proceeds with L-arginine (L-Arg), O_2, and NADPH as substrates [19]. Unlike endothelial NOS (eNOS), iNOS is Ca^{2+}/calmodulin-independent, but there has been a report that elevated intracellular calcium affects NO production by iNOS [20]. Comparing these media, F-12 contains a 2.5 times higher amount of L-Arg but DMEM contains a 6.0 times higher amount of Ca^{2+} than the other, respectively. Therefore it seems feasible that the difference in NO production might have been caused by L-Arg and Ca^{2+}. Besides, DMEM contains higher amounts of glucose and phenol red, while F-12 contains higher ones of pyruvate and vitamins that have been reported to have some influence on iNOS activity [21,22]. However, none of them showed a noticeable effect on the expression of iNOS during macrophage activation, or production of NO during re-incubation of the activated macrophages in this study (data not shown). It should be noted that macrophages showed a reduced NADPH level during incubation in DMEM (Fig. 3D), which might have reduced iNOS activity during re-incubation, and have resulted in the decrease in NO production during activation as well as re-incubation of macrophages in DMEM (Figs. 2C and 3C).

In addition, these two media have various effects on activated macrophage phenotypes other than expression of iNOS and production of NO, because O_2^--generating activity, and expression of TNF-α and IL-1β were also different between the activated macrophages in F-12 and DMEM (Fig. 4). From other laboratories, it has been reported that J774.1 cells in DMEM grow faster, and consume glutamine and serine at higher rates than in RPMI-1640 [23], or that DMEM contains amino acids and vitamins that show antioxidative properties [24]. The results of this study also seem to suggest that some factors in F-12 and/or DMEM have cumulative effects on the activation processes in JA-4 cells. Furthermore, we obtained similar but not identical results when peritoneal macrophages from BALB/c mice were used (data not shown). These results suggest that activation of various macrophages was influenced by culture medium. In the future, we will need to analyze the molecular mechanisms of these phenomena by using the macrophages prepared from gene-manipulated mouse or knockout mouse model of genes such as iNOS and pro-inflammatory cytokines.

Mostly, only one cell-culture condition is used to investigate cell functions or mechanisms. However, in this study, we found that the different culture media showed the distinct results in the activation of macrophage. Thus, in conclusion, the choice of medium is important to understand the true characteristics of the cells and cell functions including macrophage activation.

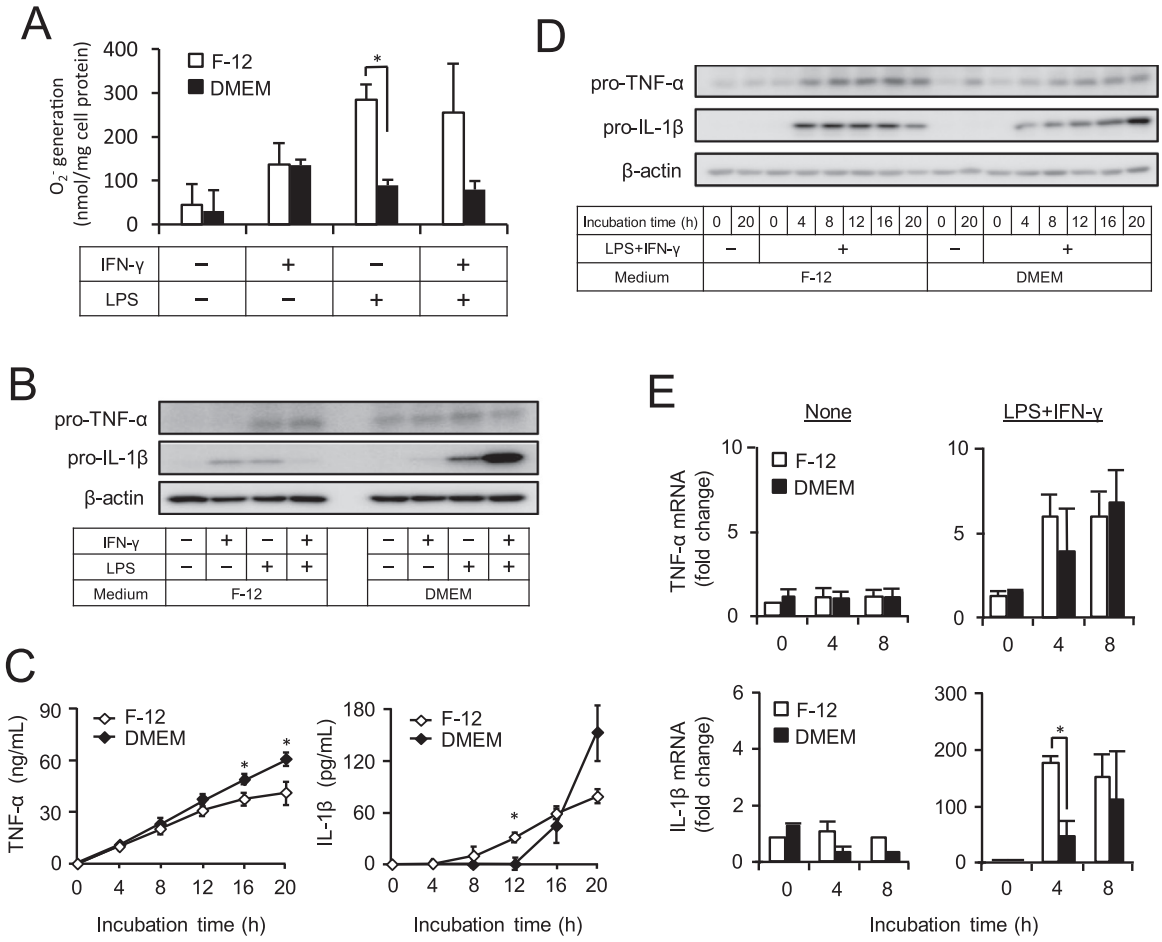

Fig. 4. Effects of culture medium on O_2^--generating activity, and induction of TNF-α and IL-1β during macrophage activation in F-12 or DMEM. O_2^--generating activity in the macrophages cultured in F-12 or DMEM with or without IFN-γ (10 units/mL) and/or LPS (100 ng/mL). (B) expression of pro-TNF-α (25 kDa) and pro-IL-1β (31 kDa) proteins detected on SDS-PAGE/western blot analysis of cell extracts after incubation in F-12 or DMEM in the presence or absence of IFN-γ and/or LPS at 37 °C for 20 h. (C) TNF-α and IL-1β release from cells cultured in F-12 or DMEM in the presence of LPS+IFN-γ. (D) time-course of induction of pro-TNF-α and pro-IL-1β proteins in macrophages treated with LPS+IFN-γ during incubation in F-12 or DMEM. (E) induction of TNF-α and IL-1β mRNA in cells cultured in F-12 or DMEM. the cells were cultured in either medium containing nothing or LPS+IFN-γ for 0, 4 or 8 h, and then mRNA was extracted and quantitated by qRT-PCR, as described in the text. the data were normalized as to GAPDH mRNA as an internal control, and the results are expressed relative to those for the untreated cells in F-12 at 0 h. in (A), (C), and (E), the results are shown as means ± S.D. for three independent experiments. $^*p < 0.05$ between indicated pairs of F-12 and DMEM.

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Appendix A. Transparency Document

Transparency Document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.01.006.

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