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Commentary Open Access

Influence of Culture Medium on Production of Nitric Oxide and Expression of Inducible Nitric Oxide Synthase by Activated Macrophages *In Vitro*

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Abstract

Activated macrophage phenotypes were influenced by the culture medium; 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) when the cells are incubated in either Ham's F-12 medium (F-12) or Dulbecco's modified Eagle medium (DMEM). Among these phenotypes, NO production and iNOS expression are the most remarkably influenced by the medium; the induction of iNOS mRNA and iNOS protein is higher in DMEM than in F-12, but NO production by activated macrophages is less in DMEM than in F-12. These results suggest that the interpretation of the experimental results requires consideration of the possibility that the differences obtained by different laboratories were caused by the culture medium used.

Keywords: Macrophage activation; Culture medium; Ham's F-12 medium; Dulbecco's modified Eagle medium; iNOS; NO production

Commentary

Macrophages play important roles in biology and pathology [1] including those in innate immune responses to pathogens, tumor cells, and apoptotic cells of the host [2-5]. Macrophages also have a unique phenotype, known as "macrophage activation," which refers to changes their properties in response to pathogen-associated molecular patterns (PAMPs), various cytokines, hormones, and other factors acting as both endogenous and exogenous stimuli, which changes occur through activation processes [1,6,7]. Among activated-macrophage phenotypes, the production of reactive oxygen species (O_2- and H_2O_2), nitric oxide (NO), and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [8-11] is the major, as well as an important, function of macrophages to exert pivotal roles in the body and to maintain homeostasis [5]. Much research on macrophage has been done by culturing primary macrophages and macrophage-like cell lines in various culture media *in vitro*. Although there have been some reports describing the effect of different culture media on the cell proliferation and differentiation of macrophages [12-14], no results have been reported precisely concerning the effects of different culture media on macrophage activation.

In a recent report [15], we showed 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) when the cells are incubated in either Ham's F-12 medium (F-12) or Dulbecco's modified Eagle medium (DMEM). For example, the production of NO, TNF-α, and IL-1β is increased more in DMEM than in F-12 after incubation of the cells continuously for 20 h; whereas the LPS-induced O_2- –generating activity is higher in F12 than in DMEM. Besides, after precise study on the mechanisms underlying the induction and expression of inducible NO synthase (iNOS) and its activity, we found that NO production and iNOS expression are the most remarkably influenced by the medium used: the induction of iNOS mRNA and iNOS protein is higher in DMEM than in F-12, but NO production by activated macrophages is less in DMEM than in F-12. iNOS is the key enzyme for the production of NO during inflammation; and it is induced especially by macrophage activation with LPS+IFN-γ, to produce NO from L-arginine (L-Arg), O_2, and NADPH as substrates [16]. Concerning Ca^{2+}, iNOS is Ca^{2+}/calmodulin independent, unlike endothelial NOS (eNOS); but there is a report that elevated intracellular calcium affects NO production by iNOS [17]. Comparing the chemical compositions of these media (Table 1), F-12 contains a 2.5 times higher amount of L-Arg than DMEM, whereas the latter contains a 6.0 times higher amount of Ca^{2+} than the former. Although it seems feasible that the difference in NO production might have been caused by the differences in L-Arg and Ca^{2+} levels in these media, our preliminary results showed that the addition of L-Arg to DMEM or that of Ca^{2+} to F-12 to adjust the concentration of each to be equal to that in the other medium failed to influence NO production from activated macrophages (data not shown). Aside from the differences in L-Arg and Ca^{2+}, DMEM contains higher amounts of glucose and phenol red; whereas F-12 contains higher ones of pyruvate and vitamins (Table 1), both of which have been reported to have some influence on iNOS activity [18,19]. However, none of them have been shown to have a noticeable effect on the expression of iNOS or production of NO (data not shown). It should be also noted that macrophages show a significantly reduced level of NADPH, a substrate of iNOS as well as NADPH oxidase, during incubation in DMEM [15], which reduction might lower iNOS activity.

Further study is necessary to determine which component of the chemical composition of these media is responsible for the altered macrophage phenotypes. We are now testing the effects of each component in these media on mouse peritoneal macrophages and RAW264.7 macrophage-like cells, especially with special interest regarding those which are at more than 2-, 10- or even 100-fold higher concentrations in one medium versus the other, as shown in Table 1.

		F-12 11765		DMEM 11965		#1 Ratio		#1 Ratio	
Components	**M.W**	**mg/L**	**mM**	**mg/L**	**mM**	**F-12/DMEM(mM)**	**$ Ratio**	**DMEM/ F-12(mM)**	**$Ratio**
Amino Acids									
Glycine	75	7.5	0.1	30	0.4	0.25		4	*
L-Alanine	89	8.9	0.1					0	
L-Arginine hydrochloride	211	211	1	84	0.398	2.51	*	0.4	
L-Asparagine-H_2O	150	15.01	0.1					0	
L-Aspartic acid	133	13.3	0.1					0	
L-Cysteine hydrochloride-H_2O	176	35.12	0.2					0	
L-Cysteine 2HCl	313			63	0.201	0			
L-Glutamic Acid	147	14.7	0.1					0	
L-Glutamine	146	146	1	584	4	0.25		4	*
L-Histidine hydrochloride-H_2O	210	21	0.1	42	0.2	0.5		2	*
L-Isoleucine	131	4	0.031	105	0.802	0.04		26.25	**
L-Leucine	131	13.1	0.1	105	0.802	0.12		8.02	*
L-Lysine hydrochloride	183	36.5	0.199	146	0.798	0.25		4	*
L-Methionine	149	4.5	0.03	30	0.201	0.15		6.67	*
L-Phenylalanine	165	5	0.03	66	0.4	0.08		13.2	**
L-Proline	115	34.5	0.3					0	
L-Serine	105	10.5	0.1	42	0.4	0.25		4	*
L-Threonine	119	11.9	0.1	95	0.798	0.13		7.98	*
L-Tryptophan	204	2.04	0.01	16	0.078	0.13		7.84	*
L-Tryosine disodium salt dihydrate	262	7.81	0.03	104	0.398	0.07		13.37	**
L-Valine	117	11.7	0.1	94	0.803	0.12		8.03	*
Vitamins									
Biotin	244	0.007	0.00003					0	
Choline chloride	140	14	0.1	4	0.029	3.5	*	0.29	
D-calcium pantothenate	477	0.5	0.001	4	0	0.13		8	*
Folic Acid	441	1.3	0.003	4	0.009	0.32		3.08	*
Niacinamide	122	0.036	0.0003	4	0.033	0.01		111.11	***
Pyridoxine hydrochloride	206	0.06	0.0003	4	0.019	0.02		66.67	**
Riboflavin	376	0.037	0.0001	0.4	0.001	0.09		10.81	**
Thiamine hydrochloride	337	0.3	0.001	4	0.012	0.08		13.33	**
Vitamin B12	1355	1.4	0.001					0	
i-Inositol	180	1.8	0.1	7.2	0.04	2.5	*	0.4	

Inorganic Salts									
Calcium Chloride ($CaCl_2$) (anhyd.)	111	33.22	0.299	200	1.802	0.17		6.02	*
Cupric sulfate ($CuSO_4$-$5H_2O$)	250	0.003	0.00001					0	
Ferric sulfate ($FeSO_4$-$7H_2O$)	278	0.834	0.003					0	
Ferric Nitrate ($Fe(NO_3)_3$"$9H_2O$)	404			0.1	0.0002	0			
Magnesium Chloride ($MgCl_2$) (anhydrous)	95	57.22	0.602					0	
Magnesium Sulfate ($MgSO_4$) (anhyd.)	120			97.67	0.814	0			
Potassium Chloride (KCl)	75	223.6	2.981	400	5.333	0.56		1.79	
Sodium Bicarbonate ($NaHCO_3$)	84	1176	14	3700	44.048	0.32		3.15	*
Sodium Chloride (NaCl)	58	7599	131.017	6400	110.345	1.19		0.84	
Sodium Phosphate monobasic (NaH_2PO_4-H_2O)	138			125	0.906	0			
Sodium Phosphate dibasic (Na_2HPO_4) anhydrous	142	142	1					0	
Zinc sulfate ($ZnSO_4$-$7H_2O$)	288	0.863	0.003					0	
Other Components									
D-Glucose (Dextrose)	180	1802	10.011	4500	25	0.4		2.5	*
Hypoxanthine Na	159	4.77	0.03					0	
Linoleic Acid	280	0.804	0.0003					0	
Lipoic Acid	206	0.21	0.001					0	
Phenol Red	376.4	1.2	0.003	15	0.04	0.08		12.5	**
Putrescine 2HCl	161	0.161	0.001					0	
Sodium Pyruvate	110	110	1					0	
Thymidine	242	0.7	0.003					0	
(Data modified from Gibco, Thero Fisher Scientific catlog) ($ Remarkable Ratios are shown as * >2.0, ***>10.0, and ***>100.0.)									

Table 1: Chemical compositions of F12 and DMED medium, and ratios of them.

Other things deserving comment are the following two: One is the importance of our findings [15] regarding performing experiments with different media. The cumulative literature concerning macrophage activation is based mainly on individual studies of researchers who have used different culture medium such as F-12, DMEM, or RPMI1640 in their macrophage-activation experiments. Therefore, the interpretation of their results seems to require consideration of the possibility that the differences obtained by different laboratories were caused by the culture medium used.

The other is the importance of our findings [15] with respect to future studies. Macrophages are located in a variety of tissues and organs as monocytes (progenitors of macrophages), tissue-specific macrophage-like Kupffer cells (liver), microglia cells (brain), alveolar macrophage (lung) [1], and also in tumors or inflammatory lesions in response to pathological stimulations. These varied distributions of macrophages seem to suggest the possibility of their exposure to different nutritional components in their micro-environments, leading to altered activation phenotypes of the macrophages. Through our study, we hope to obtain further new findings concerning the chemical components having remarkable influence on macrophage activation.

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