Cell Death Induced by Nutritional Starvation in Mouse Macrophage-like RAW264.7 Cells

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Abstract. *Amino acid utilization of mouse macrophage-like RAW264.7 cells was investigated. During the logarithmic growth stage, RAW264.7 cells grew very fast, with an approximate doubling time of 11 hours, in DMEM supplemented with 10% heat-inactivated fetal bovine serum. RAW264.7 cells consumed glutamine at the fastest rate, followed by serine, leucine, isoleucine, arginine, lysine, valine and other amino acids. When the cell density reached a critical threshold level, cells began to suffer non-apoptotic cell death characterized by mitochondrial damage (revealed by transmission electron microscopy) and a smear pattern of DNA fragmentation (revealed by agarose gel electrophoresis). At this point, glutamine, serine and glucose in the medium were almost completely exhausted, whereas other amino acids remained at more than 40% of their initial concentrations. Based on these data, it is recommended that glutamine, serine and glucose should be supplemented for the long culture of RAW264.7 cells.*

RAW264.7 cells were established from the peritoneal fluid of BALB/c mice and show the phenotype characteristics of monocytes and macrophages transformed by Abelson murine leukemia virus (1). RAW264.7 cells activated by lipopolysaccharide (LPS) have been used by numerous investigators for the study of the signal transduction pathway during the activation process of macrophages (2, 3) and the search for the substances that modify macrophage function (4). It has recently been reported that LPS induced autophagy in RAW264.7 cells (5), whereas NO donor induced apoptosis (6-8). However, much less is known about the amino acid utilization of RAW264.7 cells under different culture conditions (9). We previously reported that human myelogenous leukemic cell lines (HL-60, ML-1, KG-1), which are known to be differentiated into maturing macrophages by various differentiation-inducing agents, consumed serine at an extremely higher rate than other types of cells (10-12). Serine depletion produced the G1 arrested HL-60 cells and resulted in increasing numbers of apoptotic cells. Supplementation of serine significantly extended the period of logarithmic cell growth (10). These data suggest that the growth of the macrophage-lineage cells may depend on the extracellular concentration of serine. If this hypothesis is correct, the growth of RAW264.7 cells should also be serine dependent. To test this possibility, we investigated the consumption of various amino acids including serine during the growth of RAW264.7 cells. We also investigated the type of cell death induced by nutritional starvation in RAW264.7 cells.

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Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); trichloroacetic acid (TCA) (Wako Pure Chem Co., Tokyo, Japan).

Cell culture. Mouse macrophage-like cells RAW264.7 (kindly supplied by Prof. Ohmori, Meikai University) were cultured as an adherent cells in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% CO_2 atmosphere (4). When the cells were grown to confluency, they were collected by scraping with a rubber policeman and seeded at a lower cell density.

Determination of viable cell number. The number of viable cells was determined by hemocytometer under a light microscope after staining with 0.15% trypan blue solution.

Determination of free amino acids. Culture supernatant (medium fraction) was mixed with an equal volume of 10% TCA and stood on ice for 30 minutes. After centrifugation for 5 minutes at

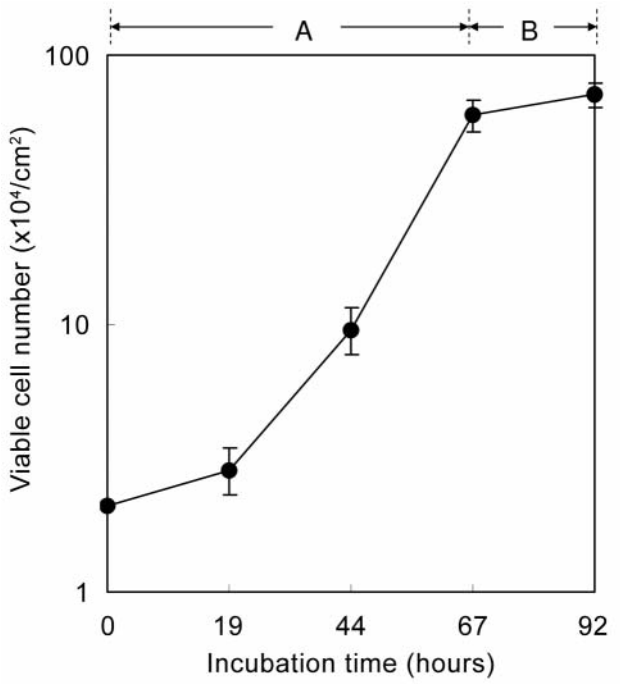

Figure 1. *Growth curve of RAW264.7 cells. Raw264.7 cells were inoculated at 2.08×10⁴/cm², cultured for the indicated times in DMEM supplemented with 10% heat-inactivated FBS without medium change, and the viable cell number was then determined. Each value represents means±S.D. from four determinants.*

Table I. *Amino acid utilization during the culture of RAW264.7 cells.*

	Amino acid concentration (μM)			Change in concentration (μM/h)	
	Initial	3 days	4 days	0-67 h	67-92 h
Consumption					
Polar, neutral					
Gln	3527±77	1101±26	253±144	–36.2	–33.9
Ser	390±9	25±2	11±2	–5.4	–0.6
Thr	720±16	614±5	591±14	–1.6	–0.9
Tyr	353±8	300±3	282±7	–0.8	–0.7
Polar, basic					
Arg	366±9	172±1	119±12	–2.9	–2.1
Lys	760±17	570±4	521±17	–2.8	–2.0
His	182±4	134±1	123±5	–0.7	–0.4
Non polar					
Leu	731±16	419±8	431±24	–4.7	0.5
Ile	732±17	532±4	542±22	–3.0	0.4
Val	731±17	568±4	534±18	–2.4	–1.4
Met	178±4	106±1	81±6	–1.1	–1.0
Cys	158±3	89±0.4	60±4	–1.0	–1.2
Phe	361±8	295±2	276±7	–1.0	–0.8
Gly	411±10	388±1	262±18	–0.3	–5.0
Production					
Non polar					
Ala	96±2	722±14	1144±53	9.3	16.9
Glu	106±10	223±5	247±2	1.7	1.0

Experimental conditions were the same as those given in Figure 1. Each value represents mean±S.D. from 4 determinants.

10,000×g, the deproteinized supernatant was collected and stored at –30˚C. The supernatants (20 μl) were subject to a JEOL JLC-500/V amino acid analyzer and amino acids were detected using the ninhydrin reaction (4).

Assay for DNA fragmentation. The cells were washed once with phosphate-buffered saline without calcium and magnesium ions [PBS(–)], and lysed with 50 μl lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsarcosinate]. The solution was incubated with 0.4 mg/ml RNase A and 0.8 mg/ml proteinase K for 2 hours at 50˚C and then mixed with 50 μl NaI solution [40 mM Tris-HCl (pH 8.0), 7.6 M NaI, 20 mM EDTA-2Na] and then 250 μl of ethanol. After centrifugation for 20 minutes at 20,000×g, the precipitate was washed with 1 ml of 70% ethanol and dissolved in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA-2Na]. Each sample (10 μl) was applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA-2Na). After staining with ethidium bromide, the DNA was visualized as described above (13). DNA from apoptotic HL-60 cells induced by UV irradiation (14) was used to locate monomer, dimer and trimer of fragmented DNA.

Electron microscopy. The cells were washed once with PBS(–), fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and collected by scraping with a rubber policeman. After standing for 1 hour at 4˚C, the cells were postfixed for one hour with 1% osmium tetraoxide-0.1 M cacodylate buffer (pH 7.4) at 4˚C, dehydrated and then embedded in Araldite 502 (CIBA-GEIGY, Basel, Switzerland). Fine sections were stained with uranyl acetate and lead citrate, and then observed under a JEM-1210 transmission electron microscope (JEOL) at an accelerating voltage of 100 kV (13).

Results

Amino acid utilization during logarithmic growth and reaching confluency. When RAW264.7 cells were inoculated at a very low cell density, they attached to the culture plate and began to rapidly proliferate during the first 67 hours (indicated by "A", Figure 1), with a doubling time of 11 hours. The growth rate of RAW264.7 cells declined when they reached the plateau phase (67-92 hours after inoculation) (indicated by "B", Figure 1).

Amino acid analysis demonstrated that RAW264.7 cells consumed glutamine at the fastest rate (36.2 μM/h), followed by serine (5.4 μM/h), leucine (4.7 μM/h), isoleucine (3.0 μM/h), arginine (2.9 μM/h), lysine (2.8 μM/h), valine (2.4 μM/h) and other amino acids (<1.6 μM/h) during the logarithmic growth phase "A". Alanine and glutamic acid were two major amino acids produced during this period (Table I). When the cells were grown to confluency, the extracellular

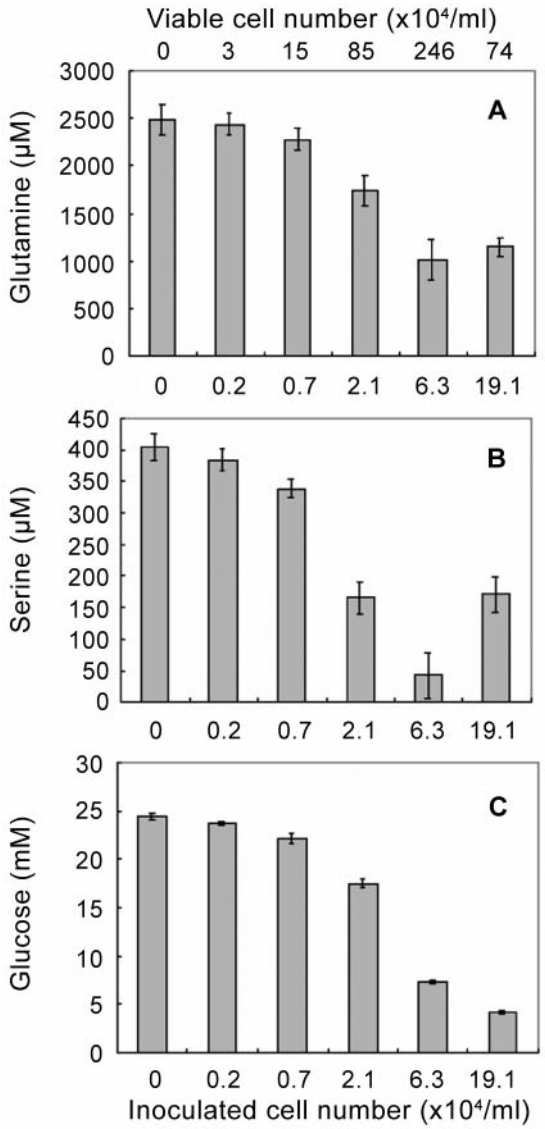

Figure 2. *Effect of increasing cell density at inoculation time on the consumption of three major nutrients for RAW264.7 cells. RAW264.7 cells (3 ml of 0, 0.2, 0.7, 2.1, 6.3 or 19.1×10⁴ cells/ml) were inoculated on 6-well plates (9.6 cm²). After incubation for 72 hours, the viable cell number (presented on top of panel A) and the extracellular concentration of glutamine (A), serine (B) and glucose (C) were determined. Each value represents means±S.D. from four determinations.*

concentration of glutamine and serine dropped to 7.1 and 2.8% of initial concentrations, respectively (declined from 3,527 to 253 μM, and 390 to 11 μM, respectively), whereas that of other amino acids was more than 40% of their initial values (Table I). It should be noted that the consumption rate of serine dropped to 0.6 μM/h during the last 25 hour (67-92 hours), as compared with glutamine (33.9 μM/h) (Table I), demonstrating that serine was the first amino acid depleted from the culture medium.

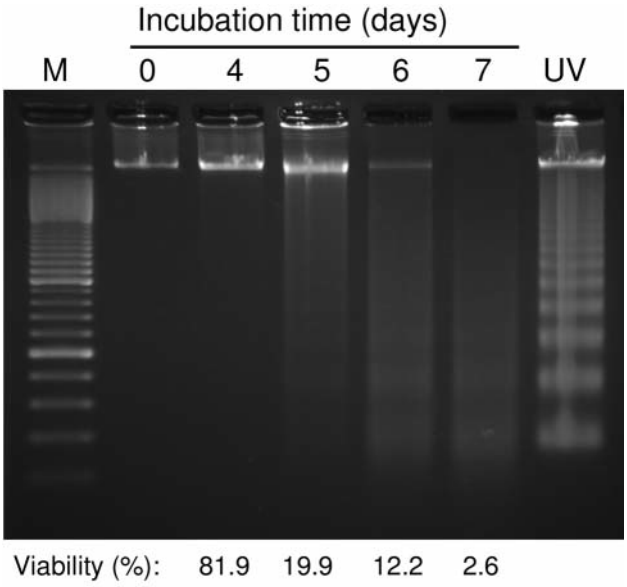

Figure 3. *Induction of smear pattern of DNA fragmentation by culturing at high cell density. Cells (5×10⁵) were inoculated onto a 6-well plate (9.6 cm²) and incubated for 0, 4, 5, 6, or 7 days, and the cell viability (%) and DNA fragmentation were then assayed. M denotes DNA marker. UV denotes the DNA from apoptotic HL-60 cells induced by UV-irradiation.*

The consumption of glutamine, a well known energy supplier in addition to glucose (15) and serine was compared in another experiment, where cells were inoculated at different cell densities (Figure 2). The consumption rate of these nutrients increased as a function of cell density. Serine was again first exhausted from the culture medium (B), followed by glucose (C) and glutamine (A) (Figure 2). It should be noted that the inoculation of the cells at an extremely high cell density resulted in cell death (viable cell number declined from 246 to 74×10⁴/ml) and reduced the consumption of glutamine (A) and serine (B) (leaving higher amounts of these amino acid in the medium) (Figure 2). This may be due to the protein degradation accompanied by cell death.

Cell death induced at high cell density. When cell density exceeded a certain threshold, the cells began to die. Agarose gel electrophoresis showed that nutritional starvation did not induce clear-cut internucleosomal DNA fragmentation, but rather produced a smear pattern of DNA fragmentation in RAW264.7 cells (Figure 3). Transmission electron microscopy demonstrated enlarged interior matrix and irregular cristae in many of the mitochondria of RAW 264.7 cells grown at extremely high cell densities (Figure 4B). In spite of the higher cell density, cell–cell attachment of cultured cells was much less (Figure 4B) than those of control cells at a lower cell density (Figure 4A). The changes in the fine structures depended on the cell density, since at

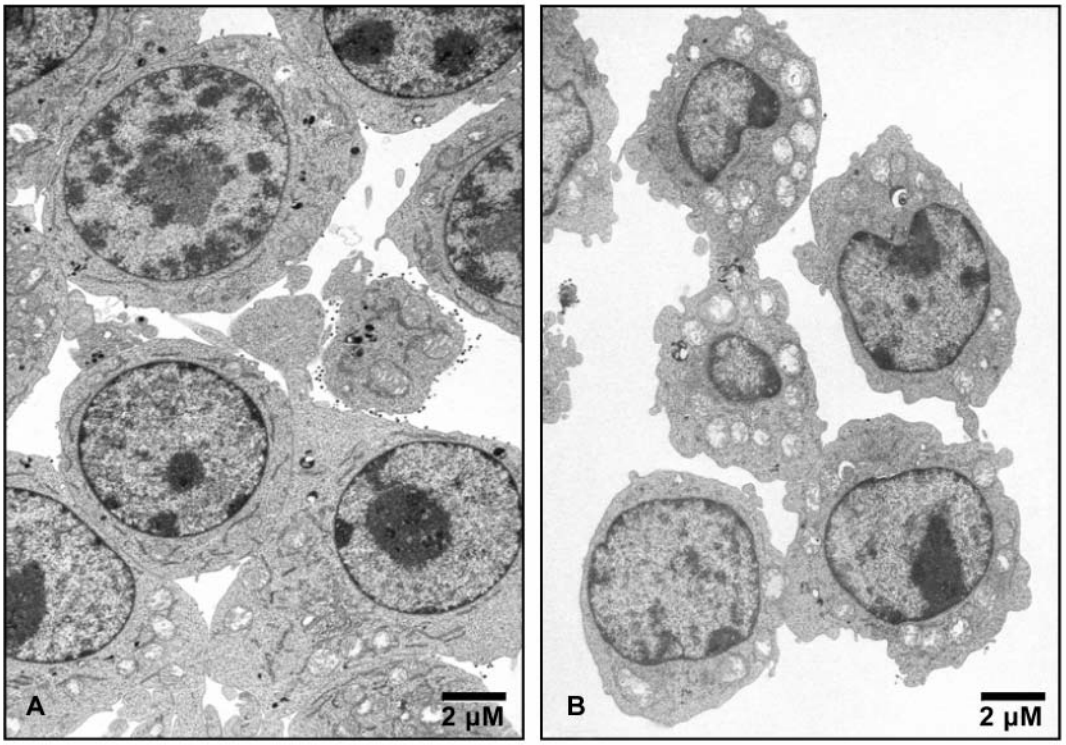

Figure 4. *Changes in the fine structure of cells during nutritional starvation. Rapidly dividing (A) and nutritionally starved RAW264.7 cells (B) were fixed and processed for transmission electron microscopy.*

lower cell densities, damage in the mitochondria was less (data not shown). These findings suggest that mitochondrial damage and lower cell–cell communication were induced by excessive cell density.

Discussion

The present study demonstrated that RAW264.7 cells consumed glutamine and serine at an extremely higher rate than other amino acids, with serine being the first amino acid to be depleted from the culture medium. This further supports our hypothesis that the growth of macrophage-lineage cells depends on serine. We also found that RAW264.7 cells were committed to non-apoptotic cell death, under nutritional starvation. This is consistent with previous reports that nutritional starvation induced autophagic cell death (characterized by formation of secondary lysosome engulfing the decomposed organelle) (16-18). Autophagy (type II programmed cell death) is a pathway for the bulk degradation of subcellular constituents through the creation of autophagosomes/autolysosomes in response to stresses such as nutrient deprivation. In general, autophagy is utilized so that cells can survive, but constitutive activation of autophagy may induce cell death (19, 20). It has been reported that LPS induced both apoptosis (21) and autophagy (5) in RAW264.7 cells. These discrepant results may be produced by differences in culture conditions. Based on the present data, it is recommended that extra glutamine, serine and glucose should be provided when RAW264.7 cells are incubated for a long time or used at a high cell density in order to prevent nutritional starvation.

Acknowledgements

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