Non-apoptotic Cell Death Induced by Nutritional Starvation in J774.1 Mouse Macrophage-like Cell Line

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Abstract. *The growth and amino acid utilization of a mouse macrophage-like cell line J774.1 was investigated in two different culture media supplemented with 10% fetal bovine serum (FBS). The J774.1 cells grew faster, and consumed glutamine and serine at higher rates in DMEM than in RPMI1640 medium. The consumption of other amino acids was much less, while considerable quantities of alanine, glutamic acid and glycine were produced by the J774.1 cells. When the cells became confluent, serine, but not glutamine, was nearly depleted from the culture medium, followed by cell death characterized by smear DNA fragmentation, slight caspase-3 activation and structural damage of the mitochondria. Serine is required for the growth of mouse macrophage-like cell lines, and DMEM is superior to RPMI1640 for long-term cell culture.*

We have recently reported that mouse macrophage-like RAW264.7 cells, established from the peritoneal fluid of BALB/c inbred mice (1) and used as a model system for the study of signal transduction during macrophage activation (2, 3), consumed glutamine and serine at a very much higher rate than other amino acids, with serine being the first amino acid to be depleted from the culture medium (4). We also have reported previously 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, also consumed serine at a much higher rate than other types of cell lines (5-7). Serine depletion induced G_1 arrest of the cell cycle and apoptosis in HL-60 cells, and serine supplementation resulted in longer logarithmic cell growth (5). These data suggest that the growth of the macrophage-lineage cells may depend on extracellular serine. If this hypothesis is correct, the growth of another popular macrophage-like cell line J774.1 established from a female BALB/c/NIH mouse as spontaneously produced type A reticulum cell sarcoma (8) and used for the study of macrophage function (9, 10), should also be serine-dependent. To test this possibility, the consumption of amino acids during the growth of J774.1 cells was investigated, and the type of cell death induced by serine starvation in J774.1 cells was also investigated.

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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); RPMI1640 (Sigma Chemical Co., St. Louis, MO, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA) and trichloroacetic acid (TCA) (Wako Pure Chem Co., Tokyo, Japan). Culture plastic dishes and plates (6-well, 24-well) were purchased from Becton Dickinson, Franklin Lakes, NJ, USA.

Cell culture. Mouse macrophage-like J774.1 cells (8) were cultured as adherent cells in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% CO_2 atmosphere (4). When the cells had grown to confluency, they were collected by splashing the medium through a thin-tip pipette or scraping with a rubber policeman and seeded at a lower cell density.

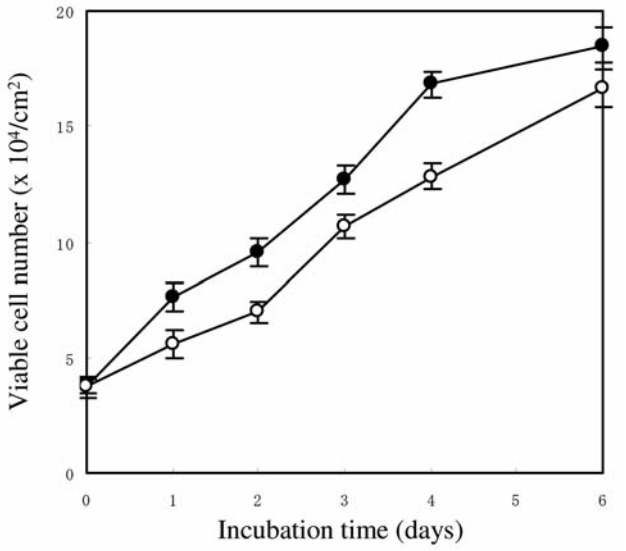

Figure 1. *Growth curve of J774.1 cells. J774.1 cells were inoculated at 3.74×10⁴/cm² on a 24-well plate. ●: DMEM, ○: RPMI-1640 each supplemented with 10% FBA. Each value represents means±S.D. from four determinations.*

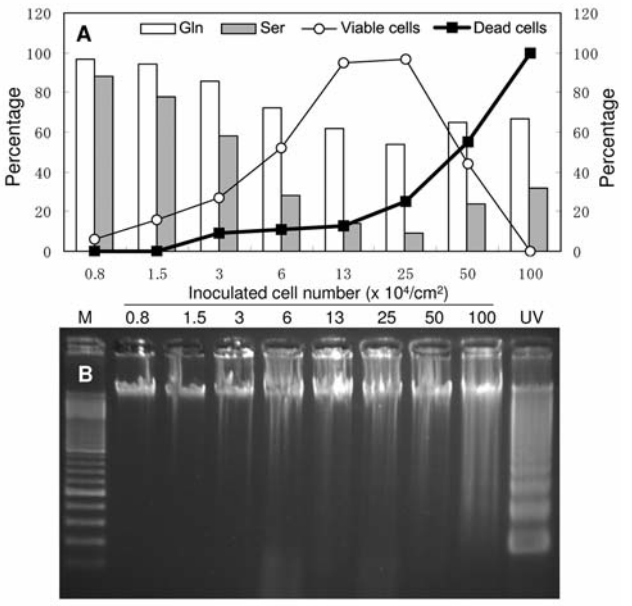

Figure 2. *Effect of inoculated cell number on cell growth (A) and DNA fragmentation (B). Cells were incubated for 4 days. (A) Viable cell number (×10⁴/cm²), % of dead cells and extracellular concentration of Gln and Ser (μM). Each value represents mean from duplicate determinations. (B) DNA fragmentation pattern on agarose gel electrophoresis. M: DNA marker, UV: DNA from apoptotic HL-60 cells induced by UV-irradiation.*

J774.1 cells were inoculated at the indicated density on a 24-well plate (Figures 1 and 2, Table I), 10-cm dish (Figure 3) or 6-well plate (Figure 4). To compare the growth promoting activity of DMEM and RPMI-1640 medium, cell culture media were replaced by fresh DMEM or RPMI-1640 supplemented with 10% FBS after 4 hours cell inoculation (Figure 1, Table I).

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 min at 21,000 xg, the deproteinized supernatant was collected and stored at –30˚C. The supernatants (20 μl) were subject to a JLC-500/V amino acid analyzer (JEOL, Tokyo) and amino acids were detected using the ninhydrin reaction (4).

Assay for DNA fragmentation. The J774.1 cells were seeded on a 24-well plate at a cell density of 0.8, 1.5, 3, 6, 13, 25, 50 or 100×10⁴ cells/cm². After incubation for 4 days, the medium was saved for amino acid determination, the cells were recovered by pipetting and the number of viable cells was determined. The cells (5×10⁴ 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) followed by 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 previously (4). DNA molecular marker (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic HL-60 cells induced by UV irradiation (6 J/m²/min, 1 min) (11) were run in parallel as positive controls. After staining with ethidium bromide, the DNA was visualized by UV irradiation, and photographed as described previously (4).

Assay for caspase-3 activation. The J774.1 cells were seeded on 10-cm dishes at a cell density of 0.28, 0.83 or 2.8×10⁴ cells/cm². After incubation for 5 days, the medium was saved for amino acid determination, the cells were washed with PBS(–) and lysed with the lysis solution. After standing for 10 min on ice and centrifugation for 5 min at 21,000 ×g, the supernatant was collected. Lysate (50 μl, equivalent to 200 μg protein) was mixed with 50 μl lysis solution containing substrates for caspase-3 (DEVD-*p*NA (*p*-nitroanilide)). After incubation for 4 hours at 37˚C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by microplate reader as described previously (12).

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 post-fixed 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

Table I. *Amino acid utilization during the culture of J774.1 cells in DMEM or RPMI-1640 medium supplemented with 10% FBS.*

Amino acids	Concentration (μM) in DMEM+10%FBS						Change in conc. (Δ μM/6 days)
	Initial	1 day	2 days	3 days	4 days	6 days	
Consumption							
Gln	3376±52	2775±94	2560±48	2090±118	1782±212	1101±94	–2275
Ser	371±3	340±5	312±11	217±18	161±43	36±8	–335
Arg	367±7	350±9	360±6	326±14	305±25	270±16	–97
Leu	733±13	705±20	730±12	677±30	653±48	654±33	–79
Cys	168±3	153±5	154±3	137±6	126±9	111±5	–57
Ile	705±13	677±19	699±13	648±27	634±44	655±32	–50
Met	182±3	177±5	185±3	170±8	161±12	152±8	–30
Val	742±13	723±19	759±12	712±30	696±50	723±32	–19
Lys	751±16	738±19	783±14	596±275	725±52	734±33	–17
His	185±4	180±5	188±3	140±72	172±14	174±7	–11
Phe	371±7	365±10	388±6	371±15	365±25	383±14	12
Tyr	349±7	345±9	366±5	351±14	346±24	361±13	12
Thr	732±9	719±20	763±16	737±30	730±49	774±29	42
Production							
Gly	394±7	400±13	443±9	446±17	440±24	452±28	58
Glu	139±8	166±1	182±5	191±32	205±6	265±10	126
Ala	84±2	132±4	230±11	405±16	576±29	1177±14	1093

Amino acids	Concentration (μM) in RPMI-1640+10%FBS						Change in conc. (Δ μM/6 days)
	Initial	1 day	2 days	3 days	4 days	6 days	
Consumption							
Gln	1442±5	1298±17	1057±36	767±59	612±128	329±158	–1113
Ser	257±3	234±7	186±13	93±9	63±41	27±16	–230
Arg	950±10	935±25	901±17	805±69	851±12	853±52	–97
Phe	155±124	173±163	88±2	72±6	71±6	73±10	–82
Lys	213±2	159±101	198±5	157±14	151±18	143±27	–70
Ile	346±5	340±11	322±8	271±23	281±21	285±48	–61
Val	179±1	175±6	164±5	129±11	126±15	126±22	–53
Thr	157±1	155±5	149±3	124±12	126±10	127±17	–30
Met	85±1	84±2	79±2	64±5	62±6	59±9	–26
Cys	167±2	166±5	161±3	144±12	153±3	151±10	–16
Leu	286±123	344±11	328±8	273±23	278±23	273±66	–13
Tyr	99±4	99±6	98±2	83±7	84±5	86±10	–13
His	91±0	120±60	88±2	76±6	79±3	80±8	–11
Production							
Gly	161±2	179±5	200±6	202±16	209±6	213±47	52
Glu	242±5	253±13	290±3	279±24	326±9	363±9	121
Ala	86±1	111±3	158±9	253±20	368±83	600±77	514

Each value represents mean±S.D. from 4 determinations.

lead citrate, and then observed under a JEM-1210 transmission electron microscope (JEOL, Tokyo) at an accelerating voltage of 100 kV as described previously (4, 12).

Results

Amino acid utilization during culture. The J774.1 cells grew slightly faster in DMEM+10% FBS than in RMPI-1640+10% FBS (Figure 1). The changes in the amino acid concentration in the culture medium during the 6 days' incubation under 5% CO_2 at 37˚C are listed in Table I. In DMEM medium supplemented with 10% FBS (upper panel, Table I), the J774.1 cells consumed glutamine at the greatest rate (2275 μM/6 days), followed by serine (335 μM), arginine (97 μM), leucine (79 μM), cysteine (57 μM), isoleucine (50 μM), methionine (30 μM), valine (19 μM), lysine (17 μM) and then histidine (11 μM). Serine was nearly depleted from the culture medium, only 9.7% of the initial concentration remained after 6 days culture. A much higher concentration (33% of the initial) of

glutamine remained. Arginine, leucine, cysteine, isoleucine, methionine, valine, lysine and histidine remained at 74, 89, 66, 93, 84, 97, 98 and 94% of the initial concentration, respectively. Alanine was the major amino acid produced by the J774.1 cells (1093 μM/6 days), followed by glutamic acid (126 μM) and then glycine (58 μM).

In RPMI-1640 medium (lower panel, Table I), glutamine was again the major amino acid consumed during the 6 days culture (1113 μM/6 days), followed by serine (230 μM), arginine (97 μM), phenylalanine (82 μM), lysine (70 μM), isoleucine (61 μM), valine (53 μM), threonine (30 μM), methionine (26 μM), cysteine (16 μM), leucine (13 μM), tyrosine (13 μM) and then histidine (11 μM). Serine was again nearly depleted from the culture medium, only 11% of the initial concentration remained after 6 days culture. Glutamine remained at 23% of the initial concentration. Arginine, phenylalanine, lysine, isoleucine, valine, threonine, methionine, cysteine, leucine, tyrosine and histidine remained at 90, 47, 67, 82, 70, 81, 69, 90, 95, 87 and 88% of the initial concentration, respectively. Alanine was again the major amino acid produced during culture (514 μM/6 days), followed by glutamic acid (121 μM) and glycine (52 μM).

It should be noted that the glutamine and serine consumption in DMEM+10% FBS was approximately 204 and 146% of that in RPMI-1640+10% FBS, respectively, in agreement with the higher growth rate in the former medium. The alanine production in DMEM+10% FBS was nearly double that in RPMI-1640+10% FBS.

Cell death induced by serine depletion. The effect of the inoculated cell number on the growth of the J774.1 cells is shown in Figure 2. When the cell number at inoculation (time 0) was in the range of 0.75 to 12.5×10^4 cells/cm^2, logarithmic cell growth was maintained for 4 days, allowing an 8.8±1.3-fold increase in the cell number. Inoculation at 25×10^4 cells/cm^2 resulted in only a 3.8-fold increase in the cell number over 4 days. Inoculation at 50×10^4 cells/cm^2 led to no increase in the cell number. Inoculation of cells at 100×10^4/cm^2 induced complete cell death (Figure 2A). The extracellular serine concentration declined with the increase in the inoculated cell number, and was lowest (9% of initial) with 25×10^4 cells/cm^2 (Figure 2A). The serine concentration was slightly higher (24, 32%) with a higher number of inoculated cells. 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 J774.1 cells (Figure 2B).

When the extracellular serine concentration dropped to 7.4 or 1.9% of the initial value as a result of increasing the inoculated cell number, the cellular caspase-3 activity increased up to 1.9 and 2.7-fold, respectively, although the extent of activation (as measured by cleavage of substrate) was much lower (only 51%) than that observed in UV-induced apoptotic HL-60 cells (Figure 3).

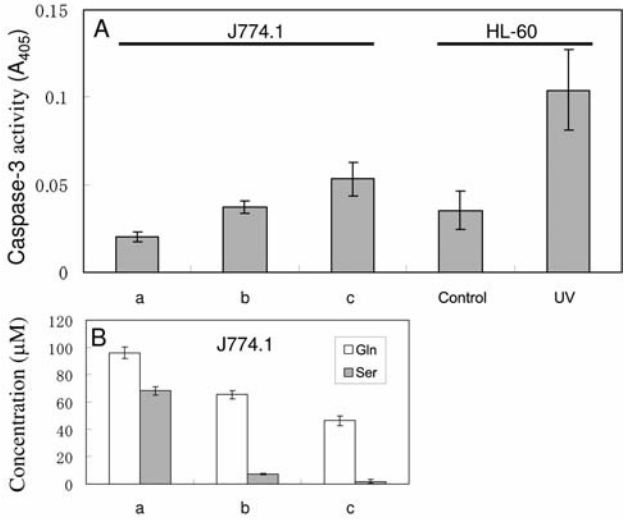

Figure 3. *Induction of caspase-3 activation. J774.1 cells were inoculated at 0.28 (a), 0.83 (b) or 2.8 (c) ×10^4/cm^2 onto 10-cm dishes, and incubated for 5 days. (A) Caspase-3 activity (absorbance at 405 nm). Cell lysate from apoptotic HL-60 cells induced by UV irradiation was used as a positive control. (B) Extracellular concentration of Gln and Ser (μM). Each value represents mean±S.D. of three determinations.*

Transmission electron microscopy demonstrated that inoculation of slightly higher numbers of cells (4.2×10^4/cm^2) induced the accumulation of lysosomes and the enlargement of mitochondria (Figure 4A and B). In the cells inoculated at a much higher cell number (12.5×10^4/cm^2), vacuolated mitochondria accompanying an electron-lucent matrix were prominent (Figure 4C), suggesting that an increase in the density of the J774.1 cells induced the structural damage of the mitochondria.

Discussion

The present study demonstrated that J774.1 cells consumed glutamine and serine at very much higher rates than other amino acids, with serine being the first amino acid to be depleted from the culture medium. The concentration of most of the other amino acids was not so much changed. This further supported our recent study with RAW264.7 cells (4) and hypothesis that the growth of macrophage-lineage cells depends on serine. Both the growth potential of the J774.1 cells and their consumption of glutamine and serine in DMEM-10% FBS were notably higher than those in RPMI-1640 10% FBS. This was probably due to the higher glutamine and serine contents of DMEM than RPMI-1640. With prolonged incubation, glutamic acid, glycine, and

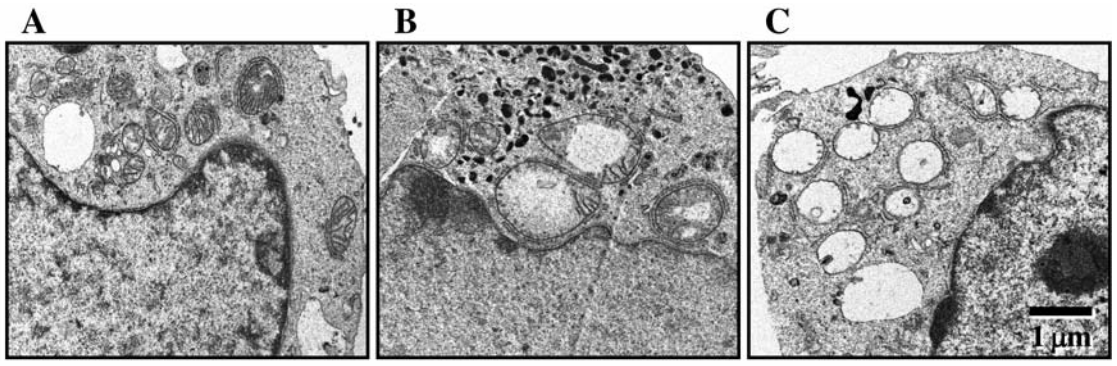

Figure 4. *Changes in the fine structure of cells during nutritional starvation. J774.1 cells were inoculated at 1.25 (A), 4.2 (B) or 12.5 (C) ×10^4/cm^2 onto 6-well plates and incubated for 4 days. Extracellular concentrations of glutamine and serine were 87±6 and 74±4% (A); 69±9 and 43±9% (B), and 79±13 and 47±9% (C) (n=3) of initial values, respectively.*

especially alanine levels, were significantly elevated. The biological significance of robust production of alanine should be elucidated.

Both the appearance of dead cells (Figure 2B) and the increase in caspase-3 activity (Figure 3) were closely associated with the depletion of serine from the culture medium. At present, it is not yet clear whether serine depletion was a direct cause of J774.1 cell death, or simply an incidental phenomenon accompanying cell death. Further studies are needed to clarify this point.

The present data suggested that serine depletion may induce non-apoptotic cell death in J774.1 cells, characterized by a smear pattern, not a laddering pattern (biochemical hallmark of apoptosis (13) induced by caspase-3 activation (14)), of DNA fragmentation (Figure 2A) and structural damage of the mitochondria (Figure 4). Further studies are necessary in order to conclude that this type of cell death is autophagic cell death (characterized by the formation of secondary lysosome engulfing the decomposed organelle). The slightly elevated glutamine and serine concentrations with the higher inoculated cell numbers might have been the result of bulk degradation of subcellular constituents in dying cells through the creation of autophagosomes/autolysosomes in response to nutrient deprivation (15, 16). In general, autophagy is utilized so that cells can survive, but constitutive activation of autophagy may induce cell death (17, 18).

Based on the present data, it is recommended that extra serine should be provided for the long-term culture of J774.1 cells to avoid nutritional starvation and cell death.

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