Changes of Caspase Activities Involved in Apoptosis of a Macrophage-Like Cell Line J774.1/JA-4 Treated with Lipopolysaccharide (LPS) and Cycloheximide

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The addition of lipopolysaccharide (LPS) together with cycloheximide (CHX) induced apoptosis in a subline of a J774.1 macrophage-like cell line, JA-4, as judged by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL)-staining and poly(adenosine 5'-diphosphate (ADP)-ribose) polymerase (PARP)-cleavage. Caspase activities were examined in these macrophages *in vitro* using fluorogenic substrates such as acetyl-DEVD-aminomethyl coumarine (Ac-DEVD-AMC, caspase-3-like), acetyl-YVAD-aminomethyl coumarine (Ac-YVAD-AMC, caspase-1-like), acetyl-VEID-aminomethyl coumarine (Ac-VEID-AMC, caspase-6-like), and carbobenzoxy-IETD-aminofluoro coumarine (Z-IETD-AFC; caspase-8-like). Kinetic studies revealed these caspase activities with different K_m and V_{max} values in extracts of apoptotic macrophages. In the course of apoptosis, caspase-3-like activity increased first at 75 min, simultaneously with the appearance of TUNEL staining and prior to PARP cleavage, and then caspase-6 and 8-like activities increased at 90 and 105 min, respectively. However, caspase-1-like activity did not change throughout the experiment. Furthermore, removal of LPS and CHX by extensive washing of the cells for 60 min completely abolished the apoptosis and the subsequent release of lactate dehydrogenase (LDH) during additional incubation until 4 h after LPS addition. However, washing of the cells after 75 min or later resulted in the progress of apoptosis and LDH release, which was coordinated with the elevation of caspase-3-like activity at 60 min and that of caspase-6 or 8-like activity at 90 min, but not with that of caspase-1-like activity. These results suggest that caspase-3-like activity represents the most apical caspase among these caspases in terms of the initiation of apoptosis in macrophages treated with LPS and CHX.

In the present study, we also provide evidence on the relatively low specificities of a series of caspase inhibitors other than acetyl-DEVD-aldehyde (Ac-DEVD-CHO) which specifically inhibited the caspase-3-like activity.

Key words apoptosis; caspase-like activity; caspase inhibitor; cytotoxicity; macrophage-like cell line; J774.1

Apoptosis is a process of cell death characterized by a series of common, biological features such as nuclear condensation and DNA fragmentation.^{1,2)} Recent progress in this field has revealed some important biochemical pathways^{3,4)} including the fragmentation of DNA into nucleosome units resulting from the activation of DNase,^{5,6)} cleavage and inactivation of poly(adenosine 5'-diphosphate(ADP)-ribose) polymerase (PARP),⁷⁾ and activation of caspase family members,^{8,9)} proteases activated in the early steps of apoptosis to cleave PARP, after which endonucleases are activated.^{10,11)}

We previously found that lipopolysaccharide (LPS) is cytotoxic toward a macrophage-like cell line, J774.1, in the presence of a protein synthesis inhibitor, cycloheximide (CHX).¹²⁾ This cytotoxicity was not induced by either LPS or CHX alone; the simultaneous addition of both was necessary.¹²⁾ The damaged cells showed remarkable release of lactate dehydrogenase (LDH) into the culture medium, which was preceded by such apoptotic changes as nuclear condensation, the formation of apoptotic bodies, DNA ladder formation, and the cleavage of 128-kDa PARP into 28-kDa fragments.¹³⁾ In order to determine which caspases are activated and trigger the apoptotic changes in macrophages treated with LPS and CHX, we assayed the activities of several caspases in macrophage extracts *in vitro*.¹⁴⁾

In this study, we precisely examined various caspase activities in apoptotic macrophages and suggest that activation of caspase-3 is most closely linked to the process of apoptosis of macrophages treated with LPS and CHX; only caspase-3-

like activity increased significantly under the conditions for the induction of the apoptotic changes, and the reagents which inhibited the caspase-3-like activity *in vitro* suppressed the apoptotic morphological changes as well as the release of LDH induced by LPS and CHX. We also provide evidence that some peptidyl inhibitors of caspases other than acetyl DEVD-aldehyde (Ac-DEVD-CHO) showed relatively low specificities to the corresponding caspase activities in apoptotic macrophages.

MATERIALS AND METHODS

Cell Culture The JA-4 line, a subline of a murine macrophage-like cell line, J774.1, was cultured in 10 ml of Ham's F-12 medium (Flow Laboratories, McLean, VA, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, U.S.A.), 50 U/ml of penicillin and 50 μ g/ml of streptomycin (Flow Laboratories) in a 100 mm plastic dish (Falcon #1001; Becton Dickinson, Lincoln Park, NJ, U.S.A.) at 37 °C in a CO₂ incubator (5% CO₂–95% humidified air), as described previously.¹²⁾

TUNEL (Terminal Deoxynucleotidyl Transferase (TdT)-Mediated Deoxyuridine (dUTP) Nick End Labeling) Staining JA-4 cells were seeded at 4×10^4 cells/100 μ l of medium into wells of a slide glass (Cel-Line Associates, Inc., U.S.A.), preincubated, and then treated in the presence or absence of 100 ng/ml LPS (LPS from *Escherichia coli* O55:B5; Sigma, St. Louis, MO, U.S.A.) and 10 μ g/ml CHX

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(Wako Pure Chemicals, Osaka, Japan). The cells were fixed directly with 3% formaldehyde in the culture medium, pH 7.0, washed with phosphate buffered saline without calcium or magnesium (PBS(-)) repeatedly, and then stained with an Apoptosis *in situ* Detection Kit (Wako Chemicals, Osaka, Japan) according to the manufacturer's protocol, as described previously.¹³⁾ Stained cells were observed under a microscope, and photographs were taken in random fields.

Assay of Cytotoxicity The cytotoxic effect of LPS on macrophages was assayed in the presence of CHX and was expressed as the release of LDH into the culture medium, as described previously.¹²⁾ LDH activity was determined with an assay kit for LDH (Kyokuto Pharmaceutical Co., Tokyo), according to the manufacturer's protocol. Cytotoxicity was expressed as % of total LDH activity released, according to the following formula:

$$\% \text{ of total} = \frac{(\text{experimental release}) - (\text{background release})}{\text{total activity}} \times 100$$

where the background release of LDH was determined by assaying the culture supernatant at zero time, and the total activity by that of cell extracts treated with 0.1% Triton X-100 at 37 °C for 30 min.¹²⁾

PARP Cleavage Assay Cleavage of PARP in the apoptotic cells was examined as described previously by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell extracts and subsequent immunoblotting using a polyclonal antibody to mouse PARP A-20 (Santa Cruz Biotechnology, CA), which recognizes the N-terminal peptide of the native PARP (MW 128 kDa) and the 28-kDa PARP fragment, but not the 85-kDa fragment.¹³⁾ The immune complexes on the membrane were reacted with [¹²⁵I] protein A and the membrane was placed on an imaging plate (Fuji Film, Tokyo),¹⁵⁾ and then both PARP and the PARP fragment were quantitated with a BAS 2000 bioimage analyzer (Fuji Film, Tokyo). The percentage of PARP fragment in total PARP, *i.e.*, PARP+PARP fragment, was calculated.

Assay of Caspase Activities Cells were seeded and treated in the presence or absence of LPS and CHX, and then scraped from the petri dishes with a cell scraper. The cells were washed once with PBS(-), and then with the extraction buffer, which comprised 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), 20 μM cytochalasin B, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 50 μg/ml antipain and 10 μg/ml chymopapain in 50 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES)-NaOH, pH 7.0,^{13,16)} by centrifugation at 10000 rpm briefly. The resultant cell pellets were suspended in 100 μl of the extraction buffer and then sonicated briefly on ice using a sonicator (Heat System). Crude extracts were prepared by five cycles of repeated freezing and thawing of the sonicated samples, centrifugation at 2000 rpm for 5 min at 4 °C, and subsequent dilution with 100 μl of the reaction buffer, which comprised 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate(CHAPS), 10 mM DTT and 0.1 mg/ml ovalbumin in 100 mM HEPES-KOH, pH 7.5.¹⁶⁾ The reaction was started by the addition of the substrate, *i.e.*, 100 μM acetyl-DEVD-aminomethyl coumarine (Ac-DEVD-AMC), 200 μM acetyl-YVAD-aminomethyl coumarine (Ac-YVAD-AMC), 200 μM acetyl-VEID-aminomethyl coumarine (Ac-VEID-AMC) (Peptide Institute.

Inc., Osaka), and 300 μM carbobenzoxy-IETD-aminofluoro coumarine (Z-IETD-AFC) (Kamiya Biomedical Company, Seattle) for caspase-3-, 1-, 6-, and 8-activities, respectively, followed by incubation at 37 °C for 2 h, or 30 min for caspase-8-like activity. After termination of the reaction by sudden chilling of the reaction mixture on ice, the fluorescence of the cleaved 7-amino-4-methyl-coumarine (AMC) or 7-amino-4-fluoro-coumarine (AFC) was measured using a spectrofluorometer (Fluoroscanner II; Labsystems Oy, Helsinki) with excitation and emission wavelengths of 355 and 460 nm for AMC, and 390 and 538 nm for AFC. The activity of each caspase protein was calculated from a standard curve for AMC or AFC and expressed in pmol of AMC or AFC cleaved per minute per milligram cell extract protein. Stoichiometrical studies for determining K_m and V_{max} values were performed by changing the substrate concentrations and by analyzing the double-reciprocal plots.

For assaying the specific effects of caspase inhibitors on the corresponding caspase activities, acetyl-YVAD-chloromethyl ketone (Ac-YVAD-CMK), acetyl-DEVD-aldehyde (Ac-DEVD-CHO), acetyl-VEID-aldehyde (Ac-VEID-CHO), carbobenzoxy-L-aspart-1-yl-[(2,6-dichlorobenzoyl)oxy] methane (Z-Asp-CH₂-DCB) (Peptide Institute Inc., Osaka) or carbobenzoxy-IETD-fluoromethyl ketone (Z-IETD-FMK) (Kamiya Biomedical Co., Seattle) was added to the enzyme assay system before adding each substrate. K_i value was determined by calculating the concentration of each caspase inhibitor that inhibited 50% of the full activity under the standard assay conditions as described above.

RESULTS

Progress of Apoptosis in Macrophages Treated with LPS and CHX The time-course study revealed that LDH release was observed at 120 min after the addition of LPS and CHX, whereas TUNEL-positive cells appeared at 75 min and cleavage of PARP was seen at 105 min (Fig. 1). These results show that disruption of the macrophage membrane was preceded by DNA nicks and cleavage of PARP (Fig. 1), suggesting that LPS and CHX induce cytotoxicity through apoptosis in macrophages.¹³⁾

Caspase Activities in Macrophages Treated with LPS and CHX We examined what kinds of caspases were involved in the apoptosis of macrophages. First of all, we tried to detect caspase-3-, 1-, 6-, and 8-like activities with specific substrates, Ac-DEVD-AMC, Ac-YVAD-AMC, Ac-VEID-AMC, and Z-IETD-AFC, respectively. All of these activities were detected in the extract of the macrophages treated with 100 ng/ml LPS and 10 μg/ml CHX at 37 °C for 2 h, when the macrophages were morphologically under apoptotic conditions (Fig. 1). Kinetic study revealed one or two K_m with the corresponding V_{max} values in each caspase activity (Fig. 2). They exhibited different saturation kinetics with different K_m and V_{max} values (Table 1). These results suggest that caspase-3-, 1-, 6-, and 8-like activities are actually present in apoptotic macrophages treated with LPS and CHX.

To determine the correlation between the elevation of caspase activities and the changes in the apoptosis of macrophages induced by LPS and CHX (Fig. 1), the time-course of the change in each caspase activity was examined. As shown in Fig. 3a, an increase in caspase-3-like activity

Fig. 1. Time-Courses of LPS-Induced Cell Damage and Apoptosis in the Presence of CHX

Cells were incubated with 100 ng/ml LPS and 10 μ g/ml CHX at 37 °C for the times indicated on the abscissa until 240 min, and then either the culture supernatant or cells were harvested for assaying of LDH release (●), or TUNEL staining (▲) and PARP cleavage (□), respectively, as described in the text. The results are expressed as the percentage of TUNEL positivity for more than 200 cells observed, or the means $\pm$ S.E. for three independent experiments (PARP cleavage and LDH release).

Fig. 2. Kinetic Studies on Caspase Activities in Macrophages Treated with LPS and CHX

An extract of JA-4 cells treated with 100 ng/ml LPS and 10 μ g/ml CHX at 37 °C for 120 min was used as the enzyme source. The cell extract was incubated with Ac-DEVD-AMC (a, caspase-3), Ac-YVAD-AMC (b, caspase-1), Ac-VEID-AMC (c, caspase-6), and Z-IETD-AFC (d, caspase-8) at 37 °C for 2 h (a—c) or 30 min (d), and the production of AMC (a—c) or AFC (d) was monitored over time using a Fluorescan II fluorescence spectrometer. The amount of cell extract used was 5 μ g (a), 40 μ g (b), 10 μ g (c), or 10 μ g (d). K_m values were determined from the double-reciprocal plots inserted in each figure.

was first observed 75 min after the addition of LPS and CHX, and it continued linearly until 120 min. On the other hand, caspase-1-like activity showed no change on the addition of LPS and CHX (Fig. 3b), and caspase-6- and 8-like activities increased first at 105 min after LPS and CHX addi-

Table 1. Kinetic Parameters for Caspase Activities in an *in Vitro* Assay Using an Extract of Macrophages Treated with LPS and CHX

Substrate	K_m 1 (μ M)	V_{max} 1 (p mol/mg/min)	K_m 2 (μ M)	V_{max} 2 (p mol/mg/min)
Ac-DEVD-AMC	25.00	316.5		
Ac-YVAD-AMC	0.59	0.98	102.0	67.7
Ac-VEID-AMC	91.6	504.1		
Z-IETD-AFC	4.6	34.3	94.4	246.0

The K_m and V_{max} values were calculated from the double-reciprocal plots in Fig. 2a—d.

Fig. 3. Elevation of Caspase Activities in Macrophages Treated with LPS and CHX

Cells were incubated without (open symbols) or with (closed symbols) 100 ng/ml LPS and 10 μ g/ml CHX for the times indicated on the abscissa at 37 °C. Then the cells were extracted as described in the text, and the individual caspase activities were determined with Ac-DEVD-AMC (a, caspase-3), Ac-YVAD-AMC (b, caspase-1), Ac-VEID-AMC (c, caspase-6), and Z-IETD-AFC (d, caspase-8). The results are the means $\pm$ S.E. for two or more independent experiments.

tion, but to a smaller extent (Fig. 3c, d). These results suggest that the increase in caspase-3-like activity occurs first, *i.e.* prior to that in caspase-1 or 8-like activity, and that this increase in caspase-3 is selectively induced by the addition of LPS and CHX at 75 min (Fig. 3a) almost simultaneously with the appearance of TUNEL-positive cells but before the cleavage of PARP (Fig. 1).

To determine how closely the elevation of caspase-3-like activity is correlated with the induction of apoptosis of macrophages, we performed washing-out experiments. Cells were treated with LPS and CHX at 37 °C for 0—60 min, washed thoroughly with medium to remove LPS and CHX, and then incubated in fresh medium until 120 min after the

first addition of LPS and CHX. Under these conditions, the cleavage of PARP became undetectable, while a control without washing-out exhibited about 20% cleavage (Fig. 4a), as shown previously.¹⁴ When the first incubation with LPS and CHX was continued for more than 75 min before washing-

out, the extent of PARP cleavage increased with time (Fig. 4a). Thus, incubation with LPS and CHX for more than 75 min is necessary for transduction of the signal for PARP cleavage. Of the caspase activities, the increase in caspase-3-like activity became evident at 60 min in the first incubation (Fig. 4b), while caspase-1-like activity remained unchanged (Fig. 4c), and caspase-6- and 8-like activities only increased after 90 min (Fig. 4d, e). These results show that elevation of caspase-3-like activity precedes that of caspase-6- or 8-like activity and that caspase-3-like activity is primarily important in the progression of apoptosis of macrophages treated with LPS and CHX.

The effects of various caspase inhibitors on LPS+CHX-induced macrophage cytotoxicity were also examined as the release of LDH (Table 2). Ac-DEVD-CHO and Z-Asp-CH₂-DCB partially inhibited the LDH release at 100 μ M and higher concentrations and Z-IETD-FMK, at 250 μ M and higher concentrations. However, the specificities of inhibitors other than Ac-DEVD-CHO were found to be relatively low when the inhibitory effects were examined in an *in vitro* study involving various caspase substrates (Table 2). Only Ac-DEVD-CHO showed selective inhibition, with a K_i value of 0.0043 μ M which was less than 1/5000 of the K_m value with Ac-DEVD-AMC as a substrate (Table 1). Although Ac-VEID-CHO showed a K_i value of 0.2 μ M, which was lower than the K_m value with Ac-VEID-AMC as a substrate (Table 1), it did not show selective inhibition of the other caspase activities with different substrates. These results imply ambiguous effects of so-called specific inhibitors on the corresponding caspase-like activities, or that macrophage caspase activities other than caspase-3-like activity are not simply characterized by selective substrates, as shown in Table 1. However, all the inhibitors that suppressed LDH release (Table 2) and PARP cleavage,¹³ *i.e.*, Ac-DEVD-CHO, Z-IETD-FMK and Z-Asp-CH₂-DCB, preferentially inhibited the caspase-3-like activity. It is not clear why Z-IETD-FMK, a so-called selective inhibitor of caspase-8, failed to inhibit caspase-8-like activity toward Z-IETD-AFC as a substrate (Table 1 and Fig. 2).

DISCUSSION

The results reported here suggest that a caspase-3-like protease is the key enzyme involved in the progress of apoptosis of macrophages treated with LPS and CHX. The increase in the caspase-3-like activity occurred abruptly at 75 min (Fig.

Fig. 4. Reversibility of the Cleavage of PARP, and the Increase in Caspase Activities in Macrophages on Treatment with LPS and CHX

(a) Cleavage of PARP. Cells were incubated with 100 ng/ml LPS and 10 μ g/ml CHX for the times indicated, washed three times with 6 ml of fresh medium, and then reincubated at 37 $^{\circ}$ C with 25 ml of fresh medium with no addition of LPS or CHX until 2 h after the addition of LPS and CHX at time 0. Finally, the cells were collected and analyzed for PARP cleavage as described in the text. (b) Caspase-3-like activity. Cells were treated as described in (a), and then cell extracts were prepared and caspase-3-like activity was measured with 100 μ M Ac-DEVD-AMC as the substrate. (c) Caspase-1-like activity assayed with 200 μ M Ac-YVAD-AMC as the substrate. (d) Caspase-6-like activity assayed with 200 μ M Ac-VEID-AMC as the substrate. (e) Caspase-8-like activity assayed with 300 μ M Z-IETD-AFC as the substrate.

Table 2. Effect of Caspase Inhibitors on the Activities of Caspases in the Macrophage Cell Lysates *in Vitro* and on the Cytotoxicity *in Vivo*

Inhibitors	K_i (μ M) ^{a)}				Inhibition of LDH ^{b)} release (%)		
	Caspase-1-like	Caspase-3-like	Caspase-6-like	Caspase-8-like	100 μ M	250 μ M	500 μ M
Ac-YVAD-CMK	>>10	4.9	>10	>10	n.d.	-5.0 $\pm$ 6.5	n.d.
Ac-DEVD-CHO	2.6	0.0043	0.6	4.2	18.9 $\pm$ 12.7	44.4 $\pm$ 5.8	62.3 $\pm$ 0.6
Ac-VEID-CHO	0.1	0.2	0.2	0.7	n.d.	-1.2 $\pm$ 14.7	n.d.
Z-IETD-FMK	>100	19.7	>100	>100	3.8 $\pm$ 0.0	69.9 $\pm$ 2.8	85.0 $\pm$ 1.4
Z-Asp-CH ₂ -DCB	>>10	1.1	4.1	>100	66.7 $\pm$ 2.8	79.2 $\pm$ 7.8	n.d.

a) The cell extracts of JA-4 cells, treated with 100 ng/ml LPS and 10 μ g/ml CHX at 37 $^{\circ}$ C for 2 h, were used as the enzyme source for caspase-1-, 3-, 6- or 8-like activities. In an *in vitro* assay, each inhibitor was added to the enzyme reaction mixture at various concentrations, and then the substrate for caspase-1-(200 μ M Ac-YVAD-MCA), 3-(100 μ M Ac-DEVD-MCA), 6-(200 μ M Ac-VEID-MCA) or 8-(300 μ M Z-IETD-AFC)-like protease was added and incubated at 37 $^{\circ}$ C for 2 h (caspase-1-, 3-, 6-like activities) or 30 min (caspase-8-like activity). The K_i value for each caspase activity was determined as described in the text. b) Cells were treated with 100, 250, 500 μ M inhibitors described above for 15 min, and then 100 ng/ml LPS and 10 μ g/ml CHX were added to the cultures. After a further 4 h incubation, the LDH release was examined as described in the text.

3a), before the increases in caspase-8- and 6-like activities (Fig. 3c, d), and before PARP cleavage and the release of LDH (Fig. 1). Moreover, the washing-out experiments showed that this increase in caspase-3-like activity was coordinated with the progress of PARP cleavage (Fig. 4)¹⁴ and the subsequent LDH release.¹³ In addition, Ac-DEVD-CHO, a specific inhibitor of caspase-3 with K_i values less than 1 nM for the purified caspase-3 from THP-1, osteosarcoma or chicken hepatoma cell extracts⁹ or for the recombinant active form of CPP32,¹⁷ which also specifically inhibited caspase-3-like activity *in vitro* with a K_i value of 0.0043 μ M in extracts of macrophages treated with LPS and CHX (Table 1), inhibited both the cleavage of PARP¹³ and the subsequent LDH release by the macrophages *in vivo* (Table 2).

The activation of caspase-3 has been reported to be dependent on the activation of caspase-1 in Fas-induced apoptosis.¹⁶ However, caspase-1 does not seem to be involved in the apoptotic changes of macrophages induced by LPS and CHX, because its activity did not change under the apoptotic conditions used in this study (Figs. 3, 4) although caspase-1-like activity was detectable in the macrophage extracts (Figs. 2, 3, and Table 1) and mRNA for pro-caspase-1 was seen in JA-4 cells with or without LPS-treatment (data not shown). Recently, caspase-8 (FLICE) was reported to be the most apical of the caspases in the cascade of apoptotic processes, and to activate caspase-3 directly.¹⁸ In our study, the caspase-8-like activity in the untreated control cell extracts was high, but its increase followed that in the caspase-3-like activity on incubation of macrophages with LPS and CHX (Fig. 3). Similar results were obtained in washing-out experiments and the increase in caspase-3-like activity occurred first, that of caspase-6 and 8-like activities following (Fig. 4). Although Z-IETD-FMK, an inhibitor of caspase-8, suppressed the LDH release resulting from the apoptotic pathway induced by LPS and CHX (Table 2), and the increase in the caspase-8-like activity was observed more than 2 h after LPS+CHX-treatment in these macrophages (Fig. 3), it seems unlikely, for the reasons given above, that the activation of the caspase-3-like protease in an apoptotic pathway is dependent on that of the caspase-8-like protease. Furthermore, Z-IETD-FMK also strongly inhibited the caspase-3-like activity in extracts of macrophages treated with LPS and CHX (Table 2), which might explain why Z-IETD-FMK suppresses the LDH release. Although a series of caspase inhibitors, except Ac-DEVD-CHO, showed relatively low specificities towards caspase-like activities (Table 2), even when the enzyme activities were assayed under stoichiometric conditions (Fig. 2 and Table 1) with apoptotic macrophage extracts pretreated with LPS+CHX at 37°C for 2 h (Figs. 1 and 3), these results seem to support the idea that caspase-3-like activity is involved in the early stage of apoptosis in LPS+CHX-treated macrophages.^{13,14} It is also worth mentioning that the application of such caspase inhibitors to the determination of the identity of certain caspases involved in apoptosis, may need more attention as to their specificities in different cell systems.

The caspase-6-like activity increased shortly after elevation of the caspase-3-like activity in macrophages treated with LPS and CHX (Fig. 3), and a similar change was observed in the washing-out experiments (Fig. 4). These results

suggest that caspase-6 is involved later in apoptosis in macrophages treated with LPS and CHX. These results are consistent with the idea that caspase-6 is the target of activated caspase-3,¹⁹ although the role of caspase-6 in the apoptosis of macrophages treated with LPS and CHX remains to be studied.

LPS-induced apoptosis of macrophages has been reported to result from the activation of macrophages by LPS.^{2,5} However, apoptosis in macrophages treated with LPS and CHX is independent of LPS-induced activation phenotypes such as nitric oxide production¹² and tumor necrosis factor- α (TNF- α) production (Karahashi and Amano, manuscript in preparation); rather, it seems to be due to early signals from LPS itself. We are currently studying the mechanisms by which LPS induces activation of the caspase-3-like protease in the presence of CHX.

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