# Docosahexaenoic acid enrichment can reduce L929 cell necrosis induced by tumor necrosis factor.

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Key words; docosahexaenoic acid, polyunsaturated fatty acids, tumor necrosis factor, necrosis, L929 cells.

Abbreviations: TNF, tumor necrosis factor; DHA, docosahexaenoic acid; DPAn-3, docosapentaenoic acid (n-3); EPA, eicosapentaenoic acid; AA, arachidonic acid; LA, linoleic acid; OA, oleic acid; FFA, free fatty acids; PUFA, polyunsaturated fatty acids; Act D, actinomycin D; BSA, bovine serum albumin; FCS, fetal calf serum; PI, propidium iodide

Abstract

We previously reported that docosahexaenoic acid (DHA) attenuated tumor necrosis factor (TNF)-induced apoptosis in human monocytic U937 cells (J. Nutr. 130: 1095-1101, 2000). In the present study, we examined the effects of DHA and other polyunsaturated fatty acids (PUFA) on TNF-induced necrosis, another mode of cell death, using L929 murine fibrosarcoma cells.

After preincubation with PUFA conjugated with BSA for 24 h, cells were treated with TNF or TNF + actinomycin D (Act D). Preincubation of cells with DHA enriched this polyunsaturated acid in the phospholipids and attenuated cell death induced by either TNF or TNF + Act D. When cells were treated with TNF alone, DNA laddering was not detected, and cells were coincidently stained with both annexin V-FITC and propidium iodide, indicating that the death mode was necrotic. TNF + Act D predominantly induced necrosis, although concurrent apoptotic cell death was also observed in this case. Preincubation with oleic acid, linoleic acid or 20:3(n-3) did not affect TNF-induced necrosis. Conversely, supplementation with n-3 docosapentaenoic acid (DPAn-3) or eicosapentaenoic acid (EPA) reduced necrotic cell death, but to a lesser extent in comparison with DHA. Unlike the case of U937 cell apoptosis, arachidonic acid (AA) significantly attenuated L929 cell necrosis, and 20:3(n-6) or 22:4(n-6) showed similar or less activity, respectively. Statistical evaluation indicated that the order of effective PUFA activity was DHA>DPAn-3≥EPA>AA ≈20:3(n-6)≥22:4(n-6). One step desaturation, C2 elongation or C2 cleavage within the n-6 or n-3 fatty acid group was probably very active in L929 cells, because AA, synthesized from 20:3(n-6) or 22:4(n-6), and C22 fatty acids, synthesized from AA or EPA, were preferentially retained in cellular phospholipids. These observations suggested that attenuation of TNF-induced necrosis by the supplementation of various C20 or C22 polyunsaturated fatty acids is mainly attributable to the enrichment of three kinds of polyunsaturated fatty acids, i.e. DHA, DPAn-3 or AA, in phospholipids. Among these fatty acids, DHA was the most effective in the reduction of L929 necrosis as observed in the case of U937 apoptosis. This suggests that DHA-enriched membranes can protect cell against TNF irrespective of death modes and that membranous DHA may abrogate the death signaling common to necrosis and apoptosis.

Introduction

Tumor necrosis factor (TNF) is a major pro-inflammatory cytokine with a wide variety of functions, one of which is induction of cell death (1, 2). TNF-induced cell death is exerted by TNF binding to a death domain-containing receptor, TNF receptor-1 (TNFRI) (2). The molecular mechanism of TNF-induced cell death has been of great interest because of the potential roles of TNF and TNF-related ligands in numerous physiological and pathological conditions (1 - 6), and the mechanism of intracellular death signaling, especially the one leading to apoptotic cell death, has been appreciably well characterized (2, 7 - 10). Unlike FAS, triggering of TNFR1 also elicits survival signals mainly through NF-kB activation (7 - 10), indicating that the balance between death and survival signals decides life and death in TNF stimulation. Although the mode of cell death induced by TNF appears to be apoptotic in most cases, induction of necrosis, of the type characterized as necrotic programmed cell death (11), has been observed with or without inhibition of signaling molecules for apoptosis including caspases (11 - 23). Recent findings indicate that necrosis can be a normal physiological and regulated event (11, 12, 24). Furthermore, it has been observed that both necrosis and apoptosis contribute to the pathology of tissue damage (24 - 29), and the disturbance of a fine balance between necrosis and apoptosis may be a key element in the development of some diseases. However, intracellular necrotic pathways and their regulation have not been as well clarified as those of apoptosis. Further clarification of pathways toward necrosis, a death mode classified as caspase-independent nonapoptotic cell death (30), can probably not only uncover the fundamental mechanism of intracellular death signaling, but also aid in the design of potential therapeutic strategies.

It has been suggested that various lipids and lipid metabolism enzymes, e.g. sphingolipids (31, 32), phospholipase A2 (33-35) and arachidonic acid (36, 37), play a role in the regulation of intracellular death signaling elicited by TNFR1 stimulation, although a number of questions still remain to be addressed. In our previous paper (38), we demonstrated that enrichment of docosahexaenoic acid (DHA) in membrane phospholipids attenuates human monocytic U937 cell apoptosis induced by TNF. Inhibitory activity against TNF-induced apoptosis is probably selective for this n-3 polyunsaturated fatty acid or 22-carbon polyunsaturated fatty acids, because enrichment of arachidonic acid or eicosapentaenoic acid showed no or a much reduced effect, respectively. We also previously reported the inhibitory effect of DHA on HL60 cell apoptosis induced by sphingosine (39), and similar anti-apoptotic activity of DHA has been reported using neuronal cells (40-43) or retinal photoreceptor cells (44-47). These previous reports indicated that DHA-enriched membrane phospholipids can protect against apoptotic cell death elicited by various cellular conditions and that DHA may be beneficial in protection against organ dysfunction to which apoptosis contributes. In

fact, it has been reported that DHA can protect against dendritic pathology in an Alzheimer's disease mouse model (48). However, it should be further clarified whether or not DHA exerts protective activity in such cases through a common mechanism. In addition, the effect of DHA on necrotic cell death has not been examined.

In this paper, we explored the effects of DHA and other unsaturated fatty acids on TNF-induced necrosis, another mode of cell death, using L929 murine fibrosarcoma cells. This cell line is well known to be sensitive to the necrotic action of TNF, and has been widely used to investigate the mechanism of necrotic signaling (12). Our results clearly demonstrated that DHA enrichment of L929 cells is remarkably effective in the protection against TNF-induced necrotic cell death.

## Materials and methods

### *Cell culture*

L929 murine fibrosarcoma cells (NCTC Clone 929) were obtained from the Health Science Research Resources Bank (Osaka, Japan). Cells were maintained in Dulbecco's modified Eagle's medium containing 10 % fetal calf serum, L-glutamine, and kanamycin at 37°C in a humidified atmosphere of 5 % $CO_2$.

### *Supplementation of fatty acids and TNF treatment*

DPA was from Cayman Chemical (MI, USA), and the other fatty acids were purchased from NU-CHEK PREP, INC (MN, USA) . In most experiments, fatty acids were added in the form bound to fatty acid-free bovine serum albumin (BSA) (Sigma, USA). Cells were cultured in 96-well or 6-well plates overnight and then supplemented with the BSA-conjugated fatty acids. After the 24 h preincubation with BSA-conjugated fatty acids, cells were treated with TNF (Roche, Mannheim, Germany) alone for 24 h or with TNF in the presence of 0.5 μg/mL actinomycin D (Wako, Osaka, Japan) for 6 h. For the assessment of cytotoxity of non-esterified DHA, a stock solution of DHA was prepared in ethanol and the final concentration of ethanol in the cell culture was adjusted to 0.5 %.

### *Cytotoxicity assay by spectrophotometry*

Cells were precultured overnight in 96-well plates (0.5 - 1 x $10^4$ cells/well), and further incubated with BSA-conjugated fatty acids for 24 h, followed by TNF treatment. Cell viability was assessed using the WST-8 assay kit (Dojindo, Kumamoto, Japan), in which a formasan dye is produced upon reduction of the water-soluble tetrazolium salt WST-8 by dehydrogenase activity. In some cases, apparent inhibitory rate of cell death was calculated as follows: 100 - {(A - B)/(C - D )} x 100. A is absorbance of cells without TNF-treatment (DHA +), B is absorbance of TNF-treated cells (DHA +), C is absorbance of cells without TNF-treatment (DHA -), D is absorbance of TNF-treated cells (DHA -).

### *Cytotoxicity assay using Annexin V-FITC and propidium iodide staining*

After TNF treatment, the cells were washed with PBS and the medium was changed to binding buffer [50 μL, (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4)]. Five μL of Annexin V-FITC (Molecular Probes, Inc. OR, USA) and 5 μL propidium iodide (1μg/mL, Sigma, USA) were added, and cells were incubated at room temperature for 15 min. The stained cells were observed under a fluorescent microscope and the results were recorded by digital image. The number of stained cells was counted by Micro analyzer software (Japan Poladigital, Japan). A minimum of 500 cells was counted for each sample under randomized conditions.

*Lipid analysis*

Cellular lipids were extracted according to the method of Bligh and Dyer (49). Phospholipids were separated using TLC with hexane/diethyl ether/acetic acid (50/50/1, v/v/v) as developing solvent. The bands corresponding to phospholipids were scraped from the plates. The fatty acid esters of phosopholipids were converted to methyl esters and analyzed by gas chromatography using a capillary column as described previously (38).

*Agarose gel electrophoresis for DNA fragmentation*

For the assessment of DNA fragmentation, cells were first seeded in 6-well plates ($4 \times 10^5$ cells/well), and the effect of DHA on TNF was assessed as described above. After TNF stimulation, cells were trypsinized and collected together with the floating cells in the medium. Cells were washed with PBS and lysed in lysis buffer (10 mM Tris-HCl buffer, pH 7.4, 10 mM EDTA, 0.5 % Triton X-100) for 15 min on ice. The lysate was centrifuged at 10000 x g for 10 min at 4°C. The supernatant was treated with Proteinase K (0.2mg/mL) and RNase A (0.4mg/mL), and then precipitated at -20 °C with 0.5 M NaCl and 50 % isopropanol prior to loading onto a 2.0 % agarose gel. DNA bands were visualized under UV light after staining with SYBR Green (Takara Biochemical, Ohtsu, Japan).

*Statistical analysis*

All statistical analyses were carried out using SPSS software (version 12.0; SPSS Inc., IL). After ascertaining homoscedasticity by Levene's test, one-way ANOVA was applied. For post hoc analysis, the difference of each group mean from the control value was analyzed using Dunnett's t procedure. Pairwise comparisons of individual groups were conducted using Tukey's test or Dunnett's T3 procedure. Significance of differences was defined as $p<0.05$.

Results and Discussion

We previously demonstrated that preincubation of U937 cells with DHA for 24 h attenuates TNF-induced apoptosis (38). At that time, it was also observed that a high dose of non-esterified DHA abrogated cell growth during the preincubation. It is well known that non-esterified free fatty acids (FFA) have a cytotoxic effect on cultured cells, although the susceptibility to cytotoxic activity of FFA is different in

each cell line. Therefore, we first examined the cytotoxic effect of non-esterified DHA on L929 cells. In this experiment, L929 cells were incubated in medium supplemented with 2.5, 5, and 10 % fetal calf serum (FCS), and with 0 ~ 100 μM non-esterified DHA, and the effect of DHA was assessed after 24 h incubation. L929 cells show a similar growth rate in the absence of DHA regardless of the FCS concentration, but more than 75 μM DHA resulted in cytotoxicy when administered with 2.5 % FCS (Fig. 1). The cytotoxic action of DHA was reduced at higher concentrations of FCS, and no cytotoxic activity was observed at 10 % FCS even when 100 μM DHA was added. The cytotoxic activity of DHA could partially be attenuated by the presence of growth factors in the FCS. However, the most important serum constituent to inhibit DHA toxicity is probably serum albumin, because 100 μM of bovine serum albumin (BSA)-conjugated DHA did not result in cytotoxicity even when L929 cells were cultured in 2.5 % FCS-containing medium (Fig. 1). Therefore, in the following experiments, we added BSA-conjugated DHA to L929 cells cultured in 10 % FCS-containing medium.

Table 1 shows the effect of incubation with DHA on the fatty acid composition of phospholipids in L929 cells. Supplementation with DHA in the cell culture for 24 h effectively enriched DHA in the phospholipids. Concomitantly, the composition of oleic acid was markedly reduced, and slight decreases in palmitoleic acid, arachidonic acid and docosapentaenoic acid (n-3) (DPAn-3) were observed. The levels of DHA in L929 phospholipids reflected the amount supplemented to the cultures at the concentrations of 5 ~ 25 μM DHA, and 25 μM DHA increased membranous DHA from 2.2 % to 20.3% of total fatty acids. Although DHA in phospholipids further increased by the addition of 50 μM DHA, differences between 25 μM and 50μM DHA were very small, indicating that there may be an upper limit of DHA in membrane phospholipids."

After 24 h of preincubation with various concentrations of DHA, 1 ~ 25 ng/mL TNF were added to the cultures, and the cell growth was determined after a further 24 h incubation (Fig. 2). Supplementation with DHA for 48 h did not significantly affect cell growth in the absence of TNF. TNF reduced cell growth dose-dependently by its cytotoxic activity. Such activity was significantly reduced by supplementation with DHA. A significant reduction in cytotoxicity was observed when 50 μM or in some cases 25 μM DHA was added, indicating that more than 20 % of DHA in membrane phospholipids (Table 1) may be needed to protect against TNF-induced death of L929 cells.

Fig. 3 showed the effect of the addition of 50 μM DHA on TNF- or TNF + actinomycin D (ActD)-induced cytotoxicity in a time-dependent manner for 36 h after TNF addition. Addition of 5 or 10 ng/mL TNF kept decreasing the cell number (Fig. 3A), and supplementation with DHA significantly rescued the cells from the cytotoxic action of TNF. In the case of 5 ng/mL TNF stimulation,

DHA-supplemented cells began to grow after 24 h, and the apparent inhibitory rate of cytotoxicity was 60 % after 36 h. When Act D was added to the culture, cell growth was almost completely inhibited regardless of the level of TNF addition (Fig. 3B). Moreover, a slight reduction in the cell number was observed by the addition of Act D alone, and this decrease in cell number was reduced by DHA-supplementation. The decrease in cell number caused by TNF was much faster when co-administered with Act D than in the absence of Act D, but DHA supplementation significantly reduced the cytotoxic action of TNF + Act D.

In order to reveal the mode of cell death induced by TNF, cell death was further assessed by exposure of phosphatidylserine (PS), as indicated by annexin V-FITC staining, and loss of plasma membrane integrity, as indicated by propidium iodide (PI) staining. Fig. 4 shows the staining of the cells at 6 h after the addition of TNF (Fig. 4A) or at 3 h after the addition of TNF + Act D (Fig. 4B). The cells treated with TNF alone were coincidently stained with both annexin V-FITC and PI (Fig. 4A). Neither DNA laddering, assessed by electrophoresis, nor nuclear-fragmented cells, assessed by Hoechst 33342 staining, were detected in TNF-treated cells (data not shown). These observations indicated that TNF-induced cell death is necrotic (50), and the annexin V-FITC staining indicated the intracellular detection of PS as a result of membrane permeabilization (50). DHA treatment reduced the proportion of cells stained with both annexin V-FITC and PI when compared with the addition of TNF alone (Fig. 4A). Table 2 shows the intact cell percentages vs. the initial cell number assessed by both types of staining. These values were more reliable for the assessment of TNF action than stained cell percentages, because the cells were growing and some of the dead cells were broken. Intact cell percentages were 2.4 ~ 3.0 times that of the cells without DHA supplementation at 24 h after TNF addition (Table 2A). These results indicated that DHA enrichment rescues cells from TNF-induced necrosis.

In contrast to the exposure to TNF alone, TNF + Act D induced staining with annexin V-FITC and PI at an earlier stage of incubation, and cells stained only with annexin V-FITC were observed (Fig. 4B, Table 2B). In addition, membrane blebbing was observed (data not shown), and DNA laddering and nuclear fragmentation were detected (data not shown). These results indicated that TNF + Act D induced apoptotic cell death. However, it is noteworthy that most of the annexin V-stained cells were also co-stained with PI in these conditions of stimulation. This might indicate that the secondary necrotic phase of the apoptotic process had occurred. However, DNA laddering was not so noticeable (data not shown) as that detected in U937 cells (38), and nuclear-fragmented cells assessed by Hoechst 33342 staining were less than 15% of PI-stained cells at 6 h after stimulation (data not shown), indicating that TNF + Act D must have predominantly induced necrotic cell death. As in the case of cell killing by TNF alone, DHA enrichment also increased the

intact cell number when added to TNF + Act D when assessed by either annexin V-FITC or PI staining (Table 2B). The values of [intact cell numbers assessed by PI - intact cell numbers assessed by annexin V-FITC], which were considered to mainly reflect apoptotic cell death, did not much differ between DHA-supplemented and DHA-nonsupplemented cultures irrespective of the TNF dose or incubation time. This indicates that inhibition of necrosis predominantly contributes to the attenuation of TNF + Act D-induced cell death by the enrichment of DHA. This presumption was confirmed by the observation that DHA did not clearly improve other characteristics of L929 cell apoptosis such as ladder formation and nuclear fragmentation (data not shown).

When U937 cells were used as target cells for the cytotoxic action of TNF, DHA appeared to be selective among fatty acids in the reduction of apoptosis, because enrichment with arachidonic acid (AA) did not affect TNF-induced U937 cell apoptosis, and supplementation with eicosapentaenoic acid (EPA) did not reduce the proportion of apoptotic cells as much as DHA supplementation (38). In order to reveal whether such selective action of DHA occurred in L929 cell necrosis, cells were preincubated with various unsaturated fatty acids for 24 h, and the effect on the TNF-induced growth inhibition, which was ascertained to reflect the necrotic activity of TNF, was examined in two separate experiments. In the first experiment (Table 3A), L 929 cells were preincubated for 24h with oleic acid (OA, 18:1(n-9)), AA, EPA, docosapentaenoic acid (DPA, 22:5(n-3)) or DHA. During the preincubation with these fatty acids, different growing rates were observed. OA reduced cell growth, and EPA, DPA and DHA did not change cell growth. Conversely, AA significantly accelerated cell growth. Exposure to 5 or 10 ng/mL TNF during a further 24 h incubation reduced the cell number. Supplementation with oleic acid did not affect TNF-induced growth inhibition, but AA, EPA, DPA and DHA significantly attenuated the necrotic action of TNF. Among these polyunsaturated fatty acids, DHA was the most effective in the reduction of L929 necrosis as in U937 cell apoptosis described previously (38). However, unlike U937 cell apoptosis, AA was also active in the reduction of L929 cell necrosis. In order to ascertain whether other C20 or n-6 polyunsaturated fatty acids show similar activity, linoleic acid (LA, 18:2(n-6)), 20:3(n-3), 20:3(n-6) and 22:4(n-6) were supplemented to L929 cell culture for 24 h (Table 3B). Only AA significantly accelerated cell growth during preincubation as observed in the first experiment. Subsequent TNF addition revealed that supplementation with LA and 20:3(n-3) did not affect the induction of necrosis by TNF (Table 3B). Conversely, 20:3(n-6) and 22:4(n-6) attenuated necrotic cell death, but the extent of attenuation by these fatty acids was significantly less than that of DHA supplementation. Data shown in Table 3A and 3B indicated that the order of fatty acids in their ability to attenuate TNF-induced L cell necrosis was as follows; DHA>DPAn-3≥EPA>AA≈20:3(n-6)≥22:4(n-6).

Table 4 shows the fatty acid levels in L929 cell phospholipids after a 24 h preincubation with various fatty acids. Supplementation with 50 μM of each unsaturated fatty acid to the cell culture enriched the corresponding fatty acid in the cells. A concomitant decrease in OA was predominant except in the case of supplementation with OA, but saturated fatty acids tended to increase. In addition to the fatty acid supplemented, it was found that the metabolites of each fatty acid increased in cellular phospholipids. When 20:3(n-6) or 22:4(n-6) was supplemented, the AA level in phospholipids increased more than or as much as the level of 20:3(n-6) or 22:4(n-6), respectively. Alternatively, supplementation with AA substantially increased 22:4(n-6), and supplementation with EPA increased the DPAn-3 in phospholipids more than twice the level of EPA in phospholipids. These indicates that one step desaturation, C2 elongation or C2 cleavage within n-6 or n-3 fatty acid group was very active in L929 cells, and that AA, synthesized from 20:3(n-6) or 22:4(n-6), and C22 fatty acids, synthesized from AA or EPA, were incorporated in cellular phospholipids. These observations suggest that attenuation of TNF-induced necrosis by supplementation with various C20 or C22 polyunsaturated fatty acids may be attributable to the enrichment of three kinds of polyunsaturated fatty acids, i.e. AA, DPAn-3 or DHA, in phospholipids. In addition, AA may have a different activity from DPAn-3 and DHA, because only AA significantly accelerated cell growth during the 24 h preincubation before TNF addition (Tables 3A and 3B).

Ingestion of dietary n-3 PUFA, including DHA, leads to their distribution to virtually every cell in the body with effects on membrane composition and function, eicosanoid synthesis, and signaling, as well as the regulation of gene expression (51). Recent observations have indicated that enrichment of DHA in membrane phospholipids can prevent apoptotic cell death of neuronal, retinal photoreceptor and leuchemic cells (38-47). Our present results demonstrated that DHA enrichment in the L929 cell membrane is also effective for protection against necrotic cell death. This suggests that DHA enrichment may reduce overall organ dysfunction caused by cell deprivation, because recent findings revealed that both necrosis and apoptosis can obviously contribute to the progress of tissue damage (24 - 29). Conversely, it has been reported that non-esterified free DHA induces apoptosis and it is speculated that this activity may contribute to the beneficial roles of n-3 fatty acids in the prevention of cancer (52). However, this speculation was mostly elicited from the evidence that FFA, including free DHA, had a cytotoxic effect on cultured cells. It has been established that FFA, when physically interacting with mitochondrial membranes, can alter the membrane permeability through an increase in proton conductance or the opening of the permeability transition pore (53). This property can explain the cytotoxic effect of FFA and indicates that fatty acid-induced cell death scarcely occurs in physiological

conditions, where most non-esterified fatty acids bind to fatty acid-binding proteins. In fact, the cytotoxic activity of free DHA against L929 cells was almost completely inhibited by albumin, a major fatty acid-binding protein in serum (54).

In this study, an appreciable amount of DHA was esterified in the phospholipids of L929 cells. Even when supplemented fatty acids were discarded by medium change after 24 h preincubation, resistance against TNF-induced necrosis was observed (data not shown), indicating that membranous DHA probably exerts the activity to reduce necrotic cell death as in the case of anti-apoptotic activity (38, 39). DPAn-3 and EPA also reduced necrotic cell death, but to a lesser extent than DHA. As discussed in U937 cell apoptosis (38), the effect of supplementation with EPA may be due to DPAn-3 enrichment in phospholipids. Supplementation with AA also significantly reduced necrotic cell death, but this was not the case in U937 apoptosis (38). The different effect of AA on L929 necrosis and U937 apoptosis might be due to a difference in signaling pathways between apoptosis and necrosis. It is unlikely that reduction of necrosis is attributable to the unsaturation rate of membrane phospholipids, because 20:3(n-3) was not active. Intracellular necrotic pathways and their regulation have not been as well clarified as those of apoptosis. Further clarification of pathways toward necrosis might elucidate the distinct role of AA. Another possible explanation is the different sensitivity to AA and/or TNF between L929 cells and U937 cells. AA or AA metabolites may have the ability to maintain L929 cell survival because AA significantly accelerated cell growth during the 24 h preincubation before TNF addition. Similar AA activity was observed in the culture of Neuro 2A cells (41).

Since supplementation with DHA had the most effective activity in the reduction of TNF-induced L929 necrosis as observed in the case of U937 apoptosis (38), it is reasonable to presume that retention of DHA in membrane phospholipids abrogates the death signaling common to necrosis and apoptosis. These two death modes show distinct morphological features, and necrosis was considered an alternative to programmed cell death for a long time. However, several studies have recently revealed that inhibition of apoptosis by caspase inhibitors can shift the type of death from apoptosis to necrosis (12, 24, 30), indicating that necrosis may be a normal physiological and programmed event. Furthermore, both necrosis and apoptosis may use some common pathways for intracellular death signaling. For instance, TNF-induced production of reactive oxygen species and the resultant sustained activation of c-Jun N-terminal kinase were required not only for necrosis but also for apoptosis (23). Necrosis could be regulated by pro-apoptotic or anti-apoptotic members of the bcl-2 family (55). Cytosolic phospholipase A2 (cPLA2), which was demonstrated to be crucial for TNF-induced necrosis of L929 cells (33), was also reported to participate in apoptotic cell death (34, 56). The possible involvement of cPLA2 in the attenuation of leuchemic cell apoptosis by DHA has

been discussed (38, 39) in relation to the inhibitory effect of DHA on cPLA2 (57). In neuronal cells, it has been proposed that DHA causes accumulation of phosphatidylserine and translocation of phosphatidylinositol 3-kinase (PI3K)/Akt, resulting in protection against apoptotic cell death induced by staurosporine (42) or serum starvation (43). This mechanism may also function for the attenuation of TNF-induced L929 cell necrosis by DHA, because PI3K/Akt might participate in necrotic cell death (58). However, at the present time, it remains to be addressed whether DHA can exert its protective activity through a mechanism common to death-inducing stimuli and various types of cells. Diverse mechanisms have been proposed for the beneficial effects of dietary n-3 PUFA (51). Further clarification of DHA behavior as a modular of cell death will provide new insight into the nutritional prevention of organ dysfunction associated with various diseases.

Acknowledgements

This work was supported by Grants- in Aid for Scientific Research (C 16500511 by E.K and C 17500537 by Y.M) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Legends to figures

Fig. 1 Effect of non-esterified DHA on L929 cell growth in various concentration of FCS

Cells (1 x $10^4$ cells) were cultured in 96-well plates overnight and incubated with the indicated concentration of non-esterified DHA or DHA-BSA conjugate for 24h. A stock solution of DHA was prepared in ethanol and the final concentration was 0.5%. Cell viability was assessed by WST-8 assay. The absorbance obtained from the initial cell number (prior to DHA treatment) was taken as 100%. Values are mean ± SD (n=6).

* $p < 0.05$ (vs. vehicle, DHA 0)

Fig. 2 Effect of DHA concentration on TNF-induced cell death

Cells (1 x $10^4$ cells) were cultured in 96-well plates overnight and incubated with the indicated concentration of DHA for 24h. After the incubation, cells were treated with TNF for 24h. Cell viability was assessed by WST-8 assay. The absorbance obtained from the initial cell number (prior to TNF treatment) was taken as 100%. Values are mean ± SD (n=5).

* $p < 0.05$ (vs. none)

Fig. 3 Effect of DHA on TNF-induced cell death in relation to the time after TNF addition

Cells (1 x $10^4$ cells) were cultured in 96-well plates overnight and incubated with DHA (50 μM) for 24h. After the incubation, cells were treated with TNF (5 or 10 ng/mL)(A) or with TNF (1 or 5 ng/mL) in the presence of Act D (0.5 μg/mL) (B). Cell viability was assessed by WST-8 assay. The absorbance obtained from the initial cell number (prior to TNF treatment) was taken as 100%. Values are mean ± SD (n=6).

* $p < 0.05$ (DHA+ vs. DHA-, within the same TNF dosage at each time point)

Fig. 4 Assessment of mode of cell death by fluorescent microscopic analysis

Cells (1 x $10^4$ cells) were cultured in 96-well plates overnight and preincubated with DHA (50 μM) for 24h. After the preincubation, cells were treated with TNF (10 ng/mL) for 6h (A) or with TNF (5 ng/mL) in the presence of actionomycin D (0.5 μg/mL) for 3h.

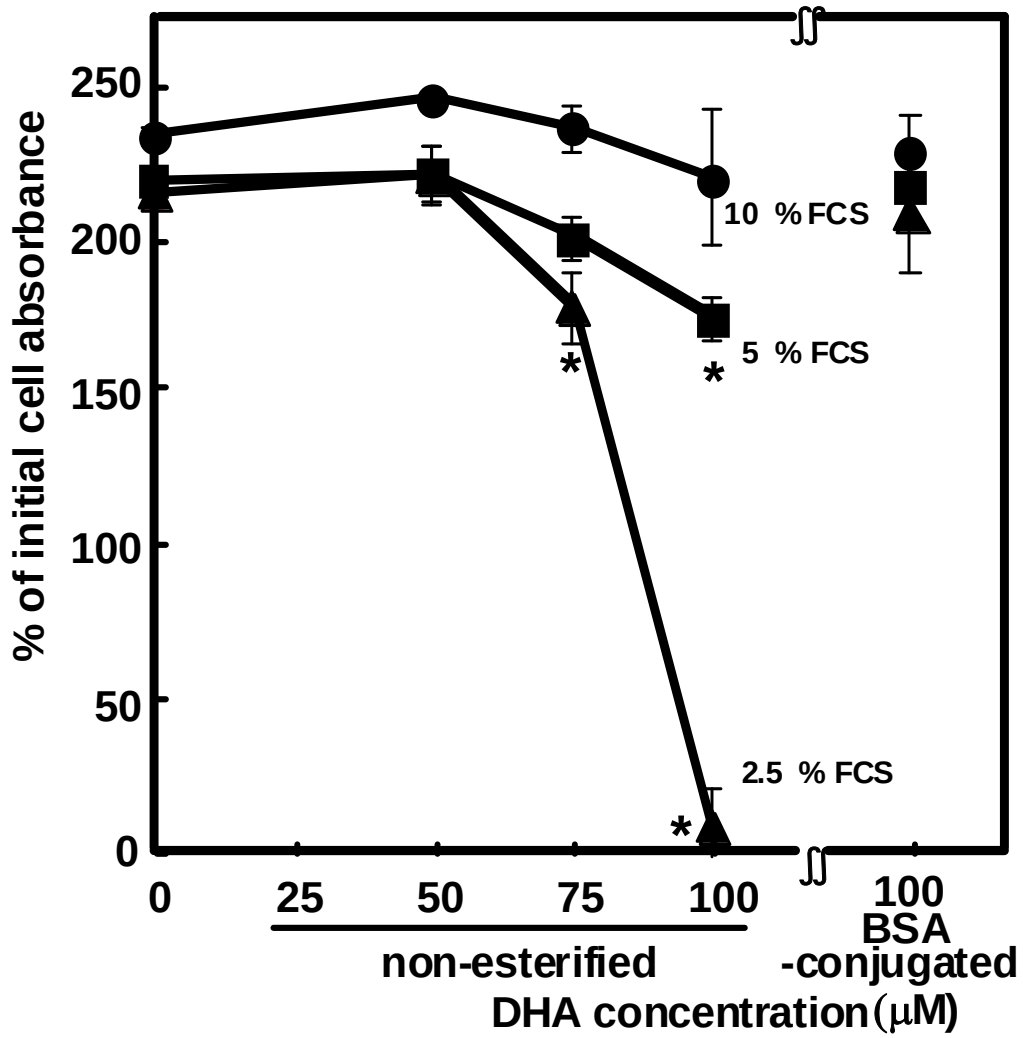
% of initial cell absorbance
250
200
150
100
50
0
10 % FCS
5 % FCS
2.5 % FCS
*
*
*
0
25
50
75
100
100
BSA
-conjugated
non-esterified
DHA concentration (μM)

Fig. 1

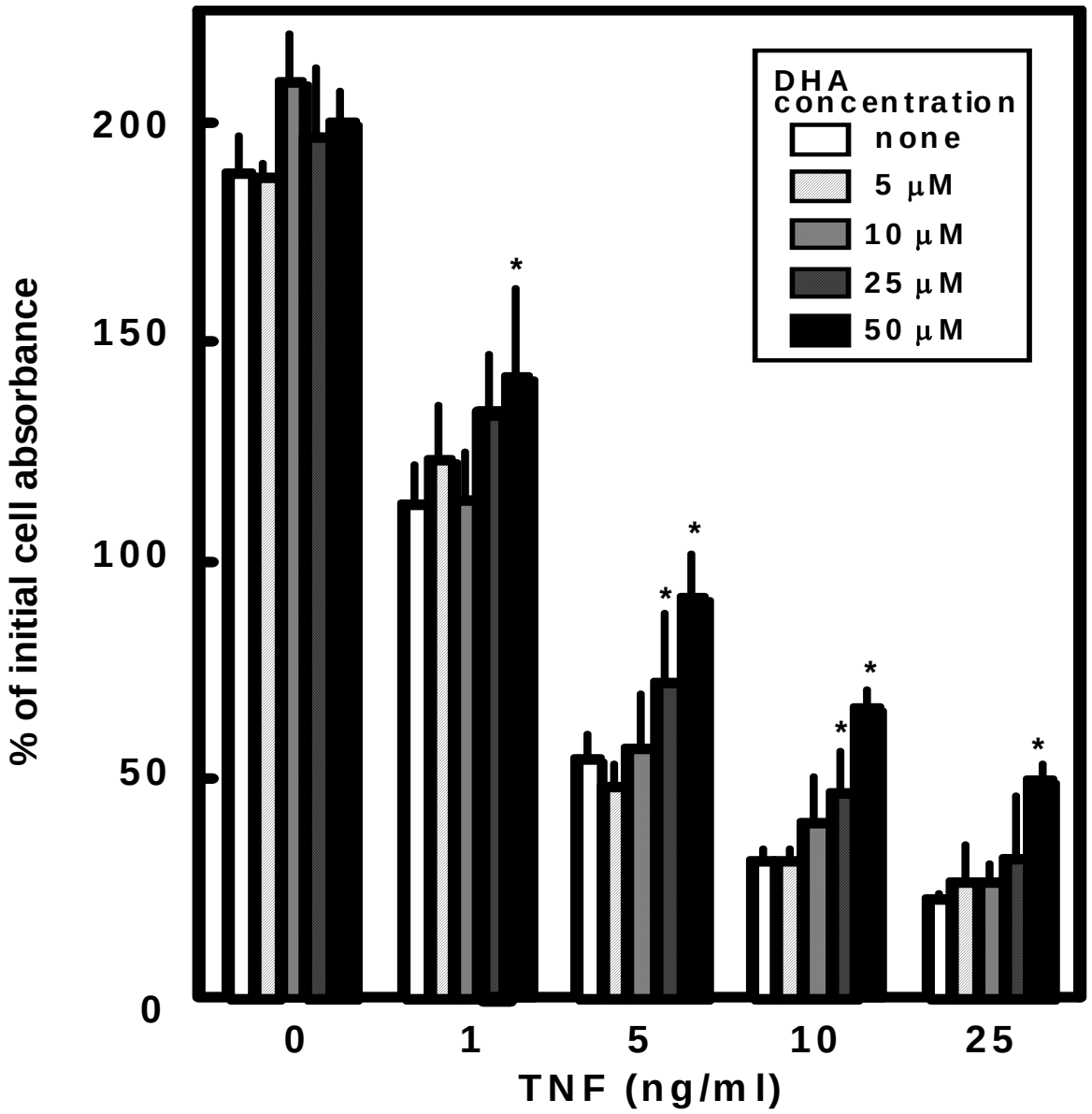
DHA concentration
none
5 μM
10 μM
25 μM
50 μM
% of initial cell absorbance
200
150
100
50
0
0
1
5
10
25
TNF (ng/ml)
*

Fig. 2

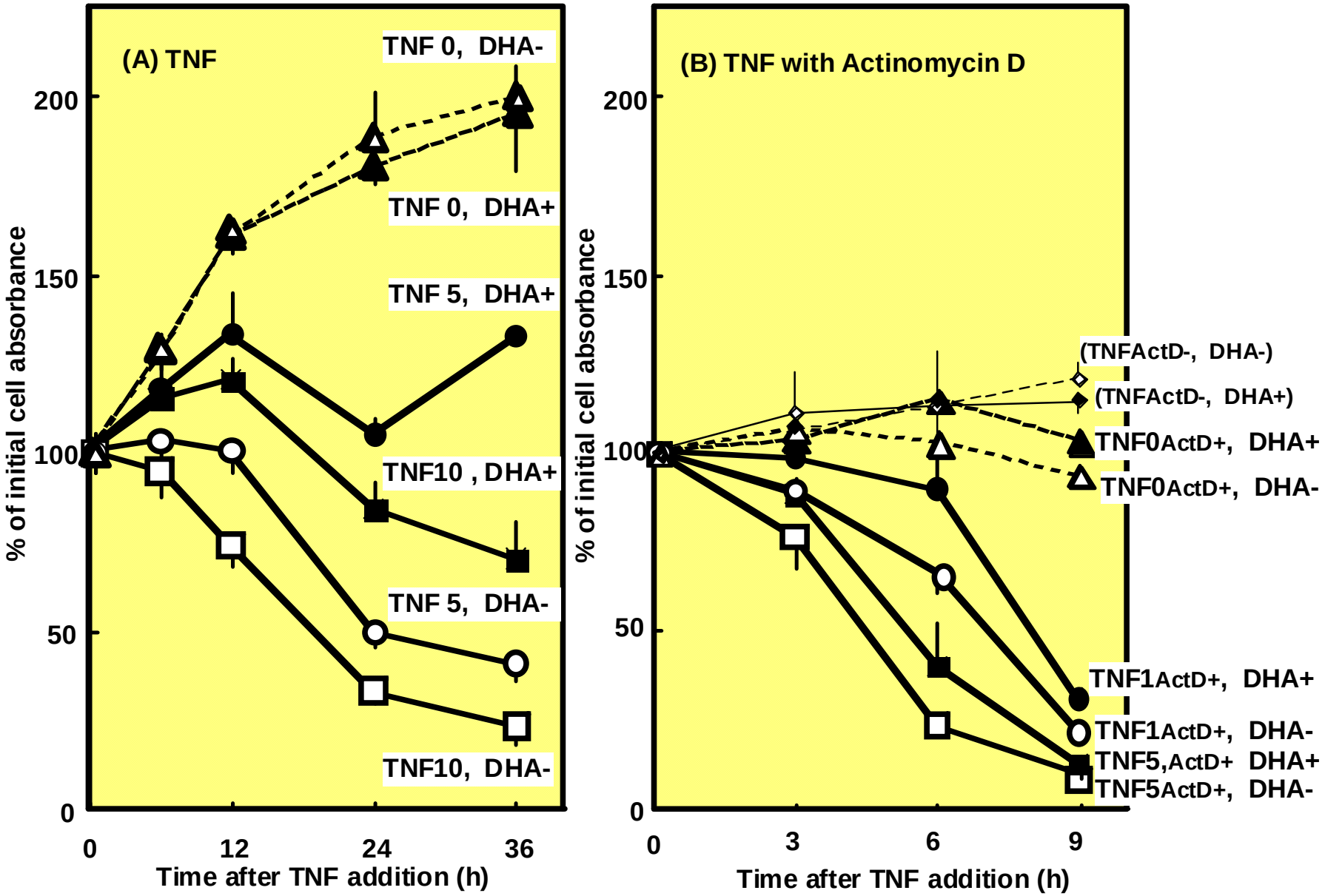
(A) TNF
TNF 0, DHA-
TNF 0, DHA+
TNF 5, DHA+
TNF10 , DHA+
TNF 5, DHA-
TNF10, DHA-
% of initial cell absorbance
200
150
100
50
0
0
12
24
36
Time after TNF addition (h)
(B) TNF with Actinomycin D
(TNFActD-, DHA-)
(TNFActD-, DHA+)
TNF0ActD+, DHA+
TNF0ActD+, DHA-
TNF1ActD+, DHA+
TNF1ActD+, DHA-
TNF5,ActD+ DHA+
TNF5ActD+, DHA-
% of initial cell absorbance
200
150
100
50
0
0
3
6
9
Time after TNF addition (h)

Fig. 3

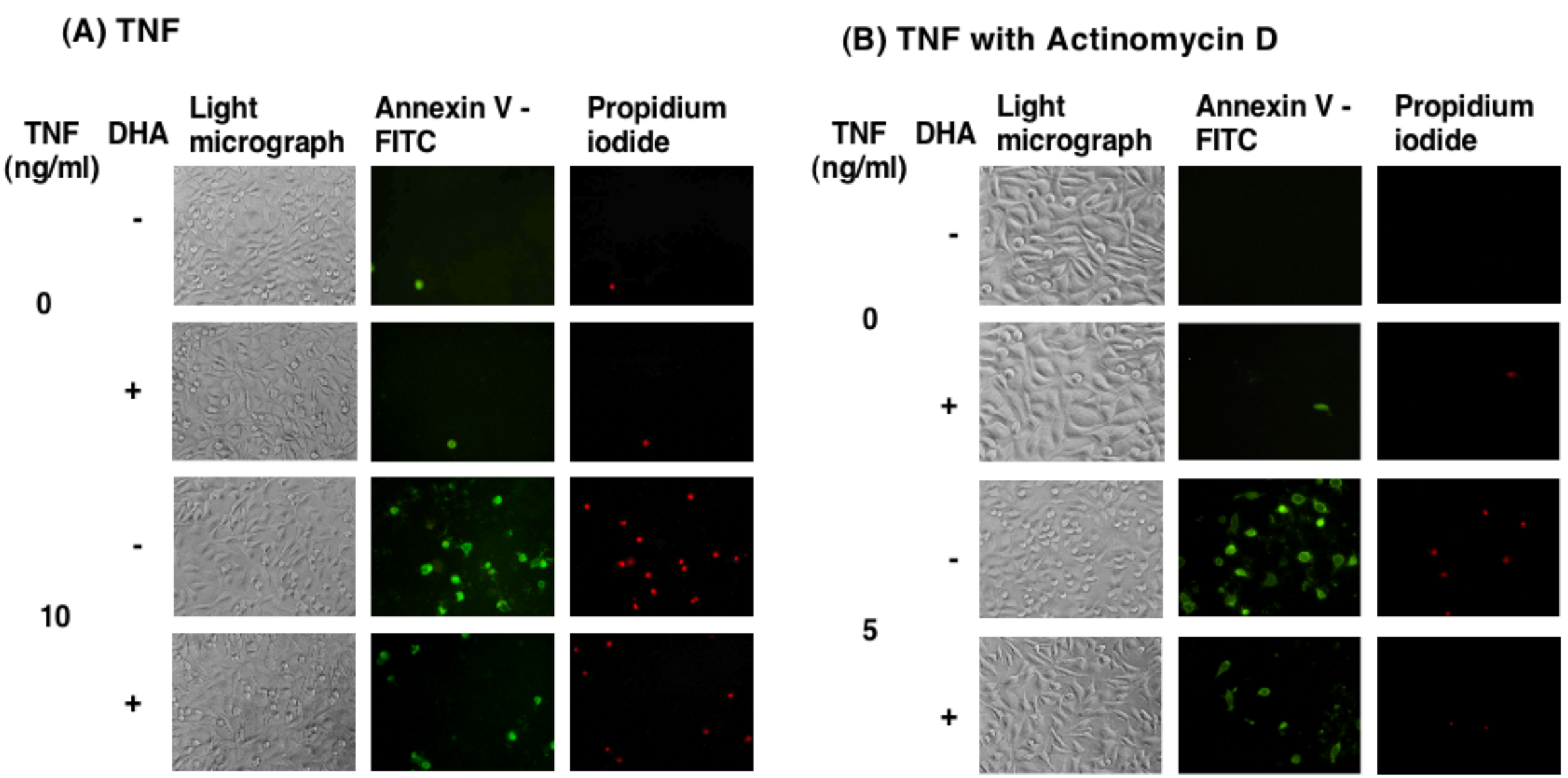
(A) TNF
TNF (ng/ml)
DHA
Light micrograph
Annexin V - FITC
Propidium iodide
0
-
+
10
-
+
(B) TNF with Actinomycin D
TNF (ng/ml)
DHA
Light micrograph
Annexin V - FITC
Propidium iodide
0
-
+
5
-
+

Fig. 4

Table 1 Fatty acid composition of phospholipid fraction from L929 cells supplemented with DHA

| Fatty Acid in Phospholipids | | Concentration of added DHA (μM) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 5 | 10 | 25 | 50 |
| | | % of total fatty acids | | | | |
| | 16:0 | 15.4 ± 0.8 | 16.7 ± 0.2* | 17.8 ± 0.1* | 18.1 ± 0.5* | 18.6 ± 0.1* |
| | 16:1 | 3.2 ± 0.2 | 2.3 ± 0.2* | 1.8 ± 0.1* | 1.4 ± 0.1* | 1.3 ± 0.1* |
| | 18:0 | 20.1 ± 0.2 | 20.7 ± 0.3* | 22.1 ± 0.1* | 23.8 ± 0.3* | 24.6 ± 0.2* |
| OA | 18:1(n-9) | 47.4 ± 0.2 | 41.1 ± 0.1* | 35.2 ± 0.1* | 26.5 ± 0.2* | 24.1 ± 0.2* |
| LA | 18:2(n-6) | 2.4 ± 0.2 | 2.1 ± 0.2 | 2.0 ± 0.0* | 1.8 ± 0.1* | 1.7 ± 0.0* |
| | 20:3(n-6) | 0.7 ± 0.0 | 0.6 ± 0.0* | 0.6 ± 0.0* | 0.6 ± 0.0* | 0.6 ± 0.0* |
| AA | 20:4(n-6) | 5.0 ± 0.2 | 4.7 ± 0.1 | 4.7 ± 0.0 | 4.1 ± 0.1* | 3.9 ± 0.1* |
| EPA | 20:5(n-3) | 0.8 ± 0.0 | 0.7 ± 0.1 | 0.7 ± 0.0 | 1.2 ± 0.1* | 1.8 ± 0.0* |
| | 22:4(n-6) | 0.4 ± 0.0 | 0.4 ± 0.0 | 0.4 ± 0.0 | 0.3 ± 0.0* | 0.3 ± 0.0* |
| DPAn-3 | 22:5(n-3) | 2.3 ± 0.1 | 2.3 ± 0.0 | 2.2 ± 0.1 | 1.9 ± 0.0* | 1.7 ± 0.0* |
| DHA | 22:6(n-6) | 2.2 ± 0.1 | 8.3 ± 0.0* | 12.8 ± 0.1* | 20.3 ± 0.3* | 21.6 ± 0.4* |

Cells were incubated with the indicated concentration of DHA. After the 24h incubation, cells were washed with PBS and subjected to lipid analyses (1 x $10^6$ cells/sample). Fatty acid are expressed as the number of carbon atoms : number of double bonds. Values (%) are mean ± SD (n=3).

* $p < 0.05$ (vs. none )

Table 2 Effects of DHA on L929 cell survival in relation to the time after addition of TNF (A) or TNF with actinomycin D (B)

| (A) | | Time after TNF stimulation | | | |
|---|---|---|---|---|---|
| | TNF | 6h | | 24h | |
| | (ng/ml) | DHA- | DHA+ | DHA- | DHA+ |
| | | % of initial cell number | | % of initial cell number | |
| Intact Cells assessed by Annexin V-FITC | 0 | 126.3 ± 0.7 | 127.8 ± 0.4* | 187.1 ± 0.3 | 178.7 ± 0.6* |
| | 5 | 92.9 ± 1.6 | 112.4 ± 1.3* | 36.0 ± 2.0 | 86.4 ± 1.3* |
| | 10 | 83.0 ± 1.7 | 108.0 ± 1.1* | 21.9 ± 2.0 | 65.3 ± 5.6* |
| Intact Cells assessed by Propidium Iodide | 0 | 126.6 ± 0.7 | 128.5 ± 0.4* | 187.6 ± 0.5 | 179.8 ± 0.3* |
| | 5 | 95.2 ± 1.8 | 113.6 ± 0.9* | 32.5 ± 2.6 | 80.4 ± 3.3* |
| | 10 | 86.4 ± 2.8 | 110.1 ± 1.8* | 20.0 ± 3.8 | 59.2 ± 3.2* |

| (B) | | Time after TNF stimulation | | | |
|---|---|---|---|---|---|
| | TNF | 3h | | 6h | |
| | (ng/mL) | DHA- | DHA+ | DHA- | DHA+ |
| | | % of initial cell number | | % of initial cell number | |
| Intact Cells assessed by Annexin V-FITC | 0 | 104.4 ± 1.7 | 101.5 ± 1.2* | 101.7 ± 0.4 | 113.2 ± 0.8* |
| | 1 | 84.3 ± 1.0 | 94.6 ± 2.1* | 51.6 ± 3.1 | 76.0 ± 3.4* |
| | 5 | 63.9 ± 2.2 | 81.4 ± 2.1* | 12.2 ± 2.1 | 27.8 ± 1.8* |
| Intact Cells assessed by Propidium Iodide | 0 | 105.4 ± 1.1 | 102.9 ± 0.5* | 102.6 ± 0.3 | 114.0 ± 0.8* |
| | 1 | 87.5 ± 0.9 | 97.8 ± 0.4* | 60.9 ± 2.3 | 85.8 ± 2.3* |
| | 5 | 72.6 ± 1.6 | 87.1 ± 0.7* | 18.9 ± 1.8 | 35.7 ± 2.2* |

Experimental procedures were described in the legend in Fig. 4. The rate of intact cells was calculated as follows: (100 – A) x B/C. A is annexin V or PI positive cells (% of remaining cells), B is cell number at the indicated time, and C is initial cell number. Cell numbers in B and C were assessed by WST-8 assay. Values are mean ± SD (n=3).

* $p < 0.05$ (DHA- vs. DHA+)

Table 3 Effect of various polyunsaturated fatty acids on TNF-induced cell death

**(A) 1st experiment**

| time after TNF addition (h) | TNF (ng/ml) | Supplementaed Fatty Acids | | | | | |
|---|---|---|---|---|---|---|---|
| | | None | OA 18:1(n-9) | AA 20:4(n-6) | EPA 20:5(n-3) | DPAn-3 22:5(n-3) | DHA 22:6(n-3) |
| | | cell absorbance (% of control) | | | | | |
| 0 | - | 100.0± 3.5 | 88.2± 4.4* | 107.4±6.2* | 104.3± 3.7 | 99.9± 4.3 | 99.9± 4.2 |
| 24 | 0 | 243.0±14.7 | 236.3±12.1 | 261.9± 2.5* | 252.6± 8.9 | 246.9±10.5 | 229.9± 9.1 |
| | 5 | 54.9± 3.7 | 64.8± 3.9 | 92.5± 3.2* | 109.9± 6.6* | 108.7±12.8* | 141.9±10.4* |
| | 10 | 47.1± 1.8 | 55.3± 3.7 | 73.1± 7.4* | 86.4±10.9* | 91.4± 1.9* | 128.2± 3.3* |

**(B) 2nd experiment**

| time after TNF addition (h) | TNF (ng/ml) | Supplementaed Fatty Acids | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | None | OA 18:1(n-9) | LA 18:2(n-6) | 20:3(n-3) | 20:3(n-6) | AA 20:4(n-6) | 22:4(n-6) | DHA 22:6(n-3) |
| | | cell absorbance (% of control) | | | | | | | |
| 0 | - | 100.0± 5.1 | 92.3± 6.9 | 93.4± 5.1 | 95.3± 6.9 | 111.0± 5.4 | 117.6±10.4* | 101.4± 9.0 | 95.0± 3.2 |
| 24 | 0 | 180.7± 7.1 | 185.6±10.8 | 172.9± 7.7 | 180.2± 8.2 | 188.1± 1.7 | 190.7± 3.6 | 194.8±12.9 | 190.0± 8.5 |
| | 5 | 78.9± 8.9 | 78.3± 4.2 | 77.1± 3.0 | 87.3± 2.8 | 116.0± 3.6* | 116.9± 6.1* | 107.2± 6.9* | 140.0± 6.8* |
| | 10 | 62.2± 6.7 | 66.6± 2.6 | 70.0± 3.6 | 69.1± 7.1 | 99.1± 2.9* | 110.2±11.2* | 83.6± 7.6* | 127.7± 9.6* |

Cells (5 x $10^3$ cells) were cultured in 96-well plates overnight and incubated with various polyunsaturated fatty acids (PUFAs, 50 μM) for 24h. After incubation, cells were treated with TNF for 24h. Cell viability was assessed by WST-8 assay. The absorbance obtained from the initial cell number (prior to TNF treatment of the control group) was taken as 100%. Values are mean ± SD (n=6). * $p < 0.05$ (vs. none)

Table 4 Fatty acid composition of phospholipids of L929 cells supplemented with various PUFAs

| Fatty Acid in Phospholipids | Supplemented Fatty Acid | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | None[1-3] | OA[1] 18:1(n-9) | LA[2] 18:2(n-6) | 20:3(n-6)[2] | 20:3(n-3)[2] | AA[1] 20:4(n-6) | EPA[1,2] 20:5(n-3) | 22:4(n-6)[2] | DPAn-3 22:5(n-3)[3] | DHA[1,3] 22:6(n-3) |
| | % of total fatty acids | | | | | | | | | |
| 16:0 | 15.9±1.5 | 12.6±0.3* | 13.6±1.3 | 15.7±0.3 | 16.2±0.2* | 17.6±1.6 | 20.6±1.4* | 18.0±1.2 | 22.4±0.4* | 18.5±1.5* |
| 16:1 | 3.4±0.4 | 1.2±0.0* | 1.0±0.1* | 1.2±0.1* | 1.6±0.1* | 1.3±0.1* | 1.5±0.1* | 1.0±0.4* | 1.5±0.1* | 1.4±0.2* |
| 18:0 | 18.3±0.2 | 13.6±0.4* | 20.2±0.2* | 20.7±0.1* | 17.6±0.1 | 22.1±0.8* | 21.3±1.1* | 20.3±0.3* | 20.9±0.2* | 23.5±0.5* |
| OA 18:1(n-9) | 48.5±2.6 | 63.0±0.7* | 22.4±0.3* | 25.5±0.1* | 28.2±0.2* | 21.1±0.7* | 21.8±1.3* | 25.8±0.2* | 20.2±0.1* | 22.9±0.5* |
| LA 18:2(n-6) | 2.2±0.2 | 1.5±0.1* | 31.0±0.1* | 1.5±0.0* | 1.7±0.0* | 1.5±0.2* | 1.6±0.1* | 1.5±0.0* | 1.6±0.1* | 1.5±0.1* |
| 18:3(n-3) | | | | | 1.5±0.1 | | | | | |
| 20:2(n-6) | | | 3.2±0.2 | | | | | | | |
| 20:3(n-6) | 0.6±0.0 | 0.4±0.0* | 0.6±0.3 | 7.8±0.2* | 0.5±0.0 | 0.4±0.0* | 0.4±0.0* | 0.4±0.1* | 0.4±0.0 | 0.5±0.0 |
| 20:3(n-3) | | | | | 13.0±0.1 | | | | | |
| AA 20:4(n-6) | 4.9±0.5 | 3.6±0.2* | 3.5±0.1* | 16.9±0.2* | 3.9±0.1* | 20.1±0.3* | 3.7±0.1* | 13.9±0.4* | 4.3±0.0* | 3.3±0.1* |
| 20:4(n-3) | | | | | 6.4±0.0 | | | | | |
| EPA 20:5(n-3) | 0.8±0.1 | 0.4±0.1 | 0.2±0.0 | 0.2±0.2 | 0.4±0.1 | 0.0±0.1* | 8.7±0.9* | 0.2±0.1 | 3.9±0.0* | 2.7±0.1* |
| 22:3(n-3) | | | | | 1.3±0.0 | | | | | |
| unknown 1 | | | | 0.9±0.2 | | | | | | |
| 22:4(n-6) | 0.6±0.1 | 0.4±0.0 | 0.6±0.1 | 5.7±0.1* | 0.4±0.1 | 12.5±0.6* | 0.4±0.1 | 15.3±0.9* | 0.3±0.1 | 0.3±0.0 |
| unknown 2 | | | | | 3.3±0.0 | | | | | |
| DPAn-3 22:5(n-3) | 2.7±0.5 | 1.7±0.1 | 2.0±0.2 | 2.2±0.1 | 2.0±0.1 | 2.0±0.3 | 19.4±1.3* | 2.0±0.1 | 23.0±0.8* | 1.6±0.1 |
| DHA 22:6(n-3) | 2.3±0.1 | 1.6±0.1 | 1.7±0.1 | 1.8±0.1 | 2.0±0.1 | 1.3±0.1* | 1.2±0.1* | 1.7±0.1 | 1.5±0.2 | 23.8±1.2* |

Cells were incubated with various PUFAs (50 μM). After the 24h incubation, cells were washed with PBS and subjected to lipid analyses (1 x $10^6$ cells/sample). Fatty acid are expressed as the number of carbon atoms : number of double bonds. Values (%) are mean ± SD, n = 8 (none), n = 6 (EPA and DHA) and n = 3 (the others).

[1-3] Lipid extracts were prepared in three separate experiments and the same superscript indicated the same batch. * $p < 0.05$ (vs. none )