short communication

Docosahexaenoic acid and arachidonic acid are fundamental supplements for the induction of neuronal differentiation

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Abstract Cell replacement therapy is being investigated for the treatment of neurodegenerative disorders. Adult autologous bone marrow-derived mesenchymal stem cells (MSCs) have been induced to differentiate into neuron-like cells harboring a variety of neuronal markers and transcription factors. Neural tissue characteristically contains high proportions of docosahexaenoic acid (DHA) and arachidonic acid (AA). In this study, evaluation of the fatty acid profile of differentiated neuron-like cells revealed a very low level of DHA, similar to that in MSCs but different from typical neurons. Supplementation of the medium with DHA alone resulted in increased levels of DHA but concomitant low levels of AA. However, supplementation with both DHA and AA yielded a fatty acid profile resembling that of neural tissue. It also resulted in enhanced outgrowth of neurite-like processes, hallmarks of neuronal differentiation. These findings demonstrate the essentiality of DHA and AA supplementation in the process of induced neuronal differentiation and have important implications for the development of cell replacement strategies of neural repair.—Kan, I., E. Melamed, D. Offen, and P. Green. **Docosahexaenoic acid and arachidonic acid are fundamental supplements for the induction of neuronal differentiation. *J. Lipid Res.* 2007.** 48: **513–517.**

Supplementary key words mesenchymal stem cells • neurite growth • cell replacement therapy • polyunsaturated fatty acids

Many neurodegenerative disorders are attributed to the degeneration of specific neuronal populations with subsequent functional loss. Although several treatments have been shown to modify the course of the disease, none successfully halted the degeneration. Owing to these factors, cell replacement therapy to replace the degenerated neural cells may serve as a valuable alternative to achieve significant clinical improvement.

Adult autologous bone marrow-derived mesenchymal stem cells (MSCs) have been studied extensively as candidates for cell replacement therapy of neurodegenerative diseases. Several laboratories, including ours, have shown that MSCs are able to differentiate beyond tissues of mesodermal origin into neuron-like cells harboring a variety of neuronal markers and transcription factors (1–7). However, none of these studies addressed the unique fatty acid composition of the neuronal membranes, namely, the high proportion of the PUFAs docosahexaenoic acid (DHA; 22:6 n-3) and arachidonic acid (AA; 20:4 n-6) (8).

DHA and AA are elongation-desaturation products of the parent fatty acids, α-linolenic acid of the omega 3 (n-3) family (18:3n-3) and linoleic acid of the omega 6 (n-6) family (18:2n-6), respectively. α-Linolenic acid and linoleic acid have been identified as essential fatty acids, because they are not synthesized de novo by animals but obtained from plants by diet. They can, however, undergo a series of elongation-desaturation steps in the mammalian organism to form the longer chain derivatives (9). In neural tissue, PUFAs are important for multiple aspects of neuronal development and function, including neurite outgrowth (10–14), signal transduction, and membrane fluidity (15–17). Although DHA is found in abundance in neuronal tissue, it cannot be synthesized by neurons and has to be supplied by the cerebrovascular endothelium and astrocytes (18).

In this study, we examined the induced differentiation of MSCs in the absence and presence of PUFA supplements.

MATERIALS AND METHODS

Isolation and culture of human MSCs

This study was approved by the Helsinki Committee of the Israel Ministry of Health and Tel Aviv University. MSCs were iso-

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; MSC, mesenchymal stem cell.

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lated as described previously (7) and cultured in growth medium consisting of DMEM (Biological Industries, Bet-Haemek, Israel) supplemented with 15% fetal calf serum (Biological Industries), 2 mM L-glutamine (Biological Industries), and 100 μg/ml streptomycin, 100 U/ml penicillin, and 12.5 U/ml nystatin (SPN; Biological Industries). The medium was replaced twice per week.

Neuronal differentiation

Neuronal differentiation was induced by a previously described protocol (7). Briefly, growth medium was replaced with differentiation medium I, consisting of DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, SPN, 10 ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN), 10 ng/ml epidermal growth factor (R&D Systems), and N2 supplement (5 μg/ml insulin, 20 nM progesterone, 100 μM putrescine, 30 nM selenium, and 100 μg/ml transferrin). Forty-eight hours later, the medium was replaced with differentiation medium II, containing DMEM supplemented with SPN, 2 mM L-glutamine, N2 supplement, 200 μM butylated hydroxyanisole (Sigma, St. Louis, MO), 1 mM dibutyryl cyclic AMP (Sigma), 3-isobutyl-1-methyl-xanthine (Sigma), and 1 μM all-*trans*-retinoic acid (Sigma), for 48 h.

Fatty acid supplementation and analysis

Various concentrations and combinations of DHA and AA, coupled with 1% horse serum and diluted in DMEM and α-tocopherol (40 μM), dissolved in ethanol, were added to differentiation medium I.

After aspiration of the medium, cultures were washed with PBS and lipids were extracted with hexane (BioLab, Jerusalem, Israel)-isopropanol (Sigma) (3:2, v/v) containing 5 mg/100 ml butylated hydroxytoluene (Sigma) as an antioxidant and 0.5 mg/100 ml heneicosanoic acid (21:0; Sigma) as an internal standard. Fatty acids were converted to fatty acid methyl esters by heating with 14% boron trifluoride in methanol (Sigma) and separated on capillary columns in an HP 5890 series II gas chromatograph equipped with a flame ionization detector. Peak areas were integrated and plotted with the aid of the Varian Star Integrator computer package. Individual fatty acid methyl esters were identified by comparing retention times with those of authentic standards. The amount of individual fatty acid was documented as the weight percentage of the total 25 identified fatty acids (mean ± SEM).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 (Sigma). Immunofluorescent double labeling was performed using mouse anti-microtubule-associated protein 2 (1:250; Zymed, San Francisco, CA) and goat anti-mouse Cy™2-conjugated AffiniPure (1:50; Jackson, West Grove, PA). Nuclei were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (Sigma).

Neurite measurements

Cells were stained with anti-microtubule-associated protein 2 antibody, to reveal the somatodendritic compartment, and 4,6-diamidino-2-phenylindole dihydrochloride nuclear stain and photographed with an Olympus IX70-S8F2 fluorescence microscope. The total neurite length per neuron was determined by measuring the individual neurite lengths with Image-Pro® Plus software (Media Cybernetics, Silver Spring, MD) and summing them per neuron. At least 20 nonclustered neuron-like cells were analyzed for each treatment group.

Statistics

The statistical significance of differences in mean neurite length between treatment groups was determined by independent-samples *t*-test.

RESULTS

Induced neuronal differentiation does not affect cellular concentrations of DHA and AA

The concentrations of DHA and AA in the differentiated neuron-like cells were 3.82 ± 0.17% and 7.17 ± 0.30%, respectively (**Fig. 1**). These values did not differ significantly from those of undifferentiated MSCs (3.00 ± 0.13% and 8.69 ± 0.63%, respectively) (Fig. 1).

DHA supplementation greatly increases DHA concentration in neuron-like cells, but at the expense of decreased AA

Supplementation of DHA at concentrations of 30, 40, 50, and 60 μM resulted in a continuous, dose-dependent increase in cellular DHA levels. However, a considerable concomitant decrease in the concentration of AA was observed (**Fig. 2**).

Combined DHA and AA supplementation results in a neuron-like cellular fatty acid composition

To prevent the enhanced decrease of AA in neuron-like cells that was triggered by DHA supplementation, we supplemented the differentiation medium with both AA and DHA. After the addition of 30, 40, and 50 μM DHA and equal concentrations, respectively, of AA, the DHA levels increased in a dose-dependent manner, whereas the concentration of AA remained stable (**Fig. 3**). The combination of 40 μM DHA and 40 μM AA resulted in a final proportion of 8.93 ± 0.24% DHA and 7.93 ± 0.24% AA in neuron-like cells. These values, as well as their ratio of

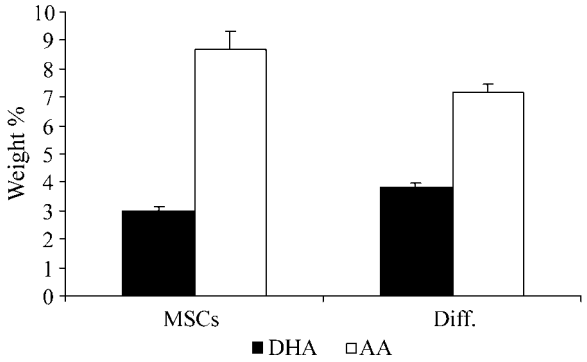

Fig. 1. Docosahexaenoic acid (DHA) and arachidonic acid (AA) concentrations in mesenchymal stem cells (MSCs) before and after induced neuronal differentiation. The fatty acid composition of the cells was determined by gas chromatography of fatty acid methyl esters, as described in Materials and Methods. MSCs indicates MSCs before induced neuronal differentiation (n = 18); Diff. indicates MSCs after induction of differentiation (n = 33). Values are expressed as weight percentage of total identified fatty acids (means ± SEM).

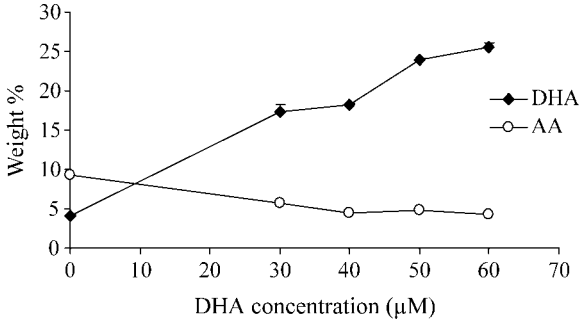

Fig. 2. DHA and AA concentrations after supplementation with DHA. Neuronal differentiation was induced either without DHA (0 μM) or with supplementation of increasing concentrations of DHA (30, 40, 50, and 60 μM), as described in Materials and Methods. The DHA and AA composition of the cells was determined by gas chromatography of fatty acid methyl esters, as described in Materials and Methods. Values are expressed as weight percentages of total identified fatty acids (means ± SEM; n = 6).

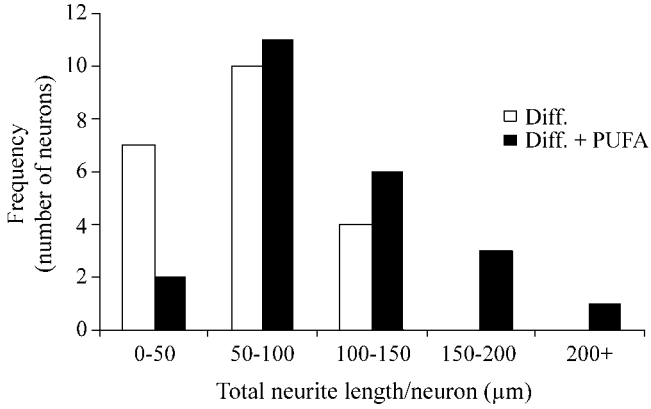

Fig. 4. Effect of DHA and AA supplementation on neurite length of differentiated cells. The neurite length of differentiated cells was determined in the absence (Diff.) and presence of 40 μM DHA and 40 μM AA (Diff.+PUFA), as described in Materials and Methods. Diff. indicates MSCs after induced neuronal differentiation.

1.13 ± 0.05, were deemed to best resemble the concentrations of these fatty acids in neural tissue. Therefore, the combination of 40 μM DHA and 40 μM AA was chosen for further experiments.

Combined DHA and AA supplementation results in enhanced neurite outgrowth

Cultures supplemented with DHA and AA during induced neuronal differentiation showed an increase in the population of neurons with longer total neurite lengths (50–200 μm and higher) and a decrease in the number of neurons with shorter total neurite lengths (0–50 μm) (**Fig. 4**). In particular, 33% of the neuron-like cells that were differentiated without PUFA supplementation had total neurite length of 0–50 μm, and 67% had total neurite length of 50–200 μm and higher. Neuron-like cells that were differentiated with PUFA supplementation had a different frequency distribution: 9% had total neurite length of 0–50 μm, and 91% had total neurite length of 50–200 μm and higher. Moreover, after PUFA supplementation, the sum of the total neurite lengths in 21 neuron-like cells increased from 1,432 to 2,514 μm. The observed difference in total neurite length per neuron between the DHA-treated neuron-like cells and the control cells was statistically significant ($P = 0.025$, by independent-samples *t*-test).

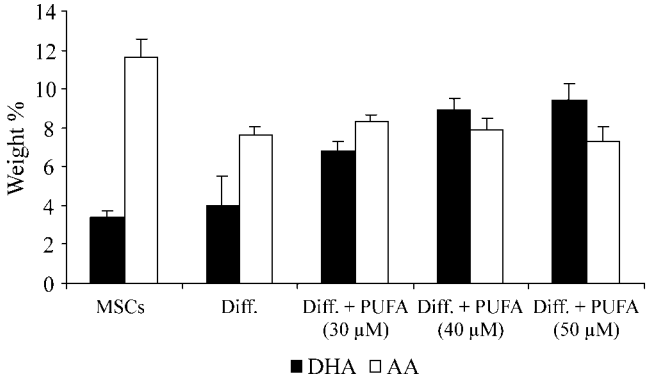

Fig. 3. DHA and AA concentrations after supplementation with both DHA and AA. Differentiation was induced either without PUFAs (Diff.) or with supplementation of different concentrations (Diff.+PUFA) of DHA and AA, as described in Materials and Methods. Diff. indicates MSCs after induced neuronal differentiation. Values are expressed as weight percentages of total identified fatty acids (means ± SEM; n = 5).

DISCUSSION

This study showed that the fatty acid profile of neuron-like cells derived from MSCs after induced differentiation was notably different from the fatty acid profile of neural tissue (19) and resembled the fatty acid profile of the MSCs. By supplementing the differentiation medium with both DHA and AA, we succeeded in attaining a cellular fatty acid profile of neural tissue. Moreover, these neuron-like cells exhibited enhanced neurite outgrowth.

We hereby report that although differentiated bone marrow-derived MSCs attain the characteristics of neuronal cells (7), they lack their typical fatty acid profile (19), the main difference being a lower level of DHA. The importance of adequate supplementation of DHA to the brain has been amply demonstrated (20). However, neurons cannot perform the final step in DHA biosynthesis and depend on its continuous supply. In this study, the neuronal induction medium contained very small amounts of AA and almost no DHA (data not shown). Supplementation of DHA increased the cellular DHA concentration but also led to a decrease in the concentration of AA. This effect of DHA supplementation has been observed previously (21). Because both PUFAs are critical to neuronal function, we decided to add DHA and AA to the differentiation medium in an attempt to achieve the typical neuronal fatty acid composition. After trials with several dose combinations, we found that supplementing the differen-

tiation medium with 40 μM DHA and 40 μM AA resulted in cellular fatty acid profile values resembling those of neural cells from the region of the striatum (19). In addition, the enriched differentiation yielded the inverted DHA/AA ratio (DHA/AA > 1) that characterizes typical neurons.

Cell cultures of neuronal origin have been supplemented previously with DHA, mainly in experiments exploring the role of DHA in neurons (13, 22). Even though increased concentrations of DHA were achieved, the basic proportions of DHA and AA did not change. This is the first time, however, that nonneuronal cells of bone marrow origin (MSCs), induced to differentiate into neurons, attained the cellular fatty acid profile that characterizes typical neurons. It should be mentioned that in the absence of the neuronal induction, MSCs did not attain a neuronal fatty acid profile after PUFA enrichment of the growth medium (data not shown). Thus, both neuronal induction and supplementation with PUFAs, especially DHA, are necessary for MSCs to attain neuronal characteristics.

One of the critical steps in neuronal differentiation is the outgrowth of neuronal processes (axons and dendrites), which establish the neuron's structural and functional polarity (23, 24). It has been demonstrated previously that in the course of neuronal induction, human MSCs develop cellular features of neuritogenesis and synaptogenesis similar to those observed in immature neurons (25). In this study, the addition of 40 μM DHA and 40 μM AA to the induction differentiation medium enhanced the neurite growth of neuron-like cells. Several studies have demonstrated both an increase in the population of neurons with longer neurites and a higher number of branches after DHA and AA supplementation, indicating a possible role of these PUFAs in promoting neuronal differentiation (10–14). In these experiments, AA and DHA at concentrations of 1.5–60 μM significantly increased neurite outgrowth in several cell types of neuronal origin. The novelty of our study lies in the fact that the same effect was observed with cells of mesenchymal origin engineered to become neurons. The molecular mechanisms underlying the contribution of DHA and AA to neurite growth are not completely understood, and several possibilities have been raised. These include stimulation of the phospholipid synthesis required for neurite elongation and membrane expansion and modulation of the signal transduction pathways involved in neurite outgrowth. Clearly, further studies are required to establish the exact mechanism(s) of PUFA-induced neurite growth in MSCs undergoing induced neuronal differentiation in vitro.

In conclusion, we have demonstrated the need for both DHA and AA supplementation to MSCs in the process of induced neuronal differentiation. The membrane composition of the differentiated cells is an important feature that influences their integration, synaptic formation, and function. Therefore, the attainment of a neuronal fatty acid profile during induced neuronal differentiation is an essential step in the induction of stem cells into functional neurons.

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