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Evidence for growth-promoting effects of Omega *n* − 3 fatty acids alone and in combination with a specific vitamin and mineral complex in rat neuroblastoma cells

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Abstract
The beneficial effects of Omega *n* − 3 polyunsaturated fatty acids (*n* − 3 PUFA) in situations of cognitive impairment may be associated with enhanced neuronal growth. Since neuronal growth is impaired in *n* − 3 PUFA deficiency, and enhanced by certain vitamins and trace elements, the effects of *n* − 3 PUFA, vitamin and mineral cell complex (VMC) and their association on neuronal growth were investigated in cultured rat neuroblastoma cells. Treatment of cells for 3 days with *n* − 3 PUFA significantly enhanced neurite length without affecting the number of neurites or cells. VMC significantly increased cell number without affecting neurite length or their number. Combined *n* − 3 PUFA and VMC significantly enhanced all three growth parameters. The data confirm the growth promoting effects of *n* − 3 PUFA and VMC in cultured neurons over a relatively short time frame.

Keywords: *Omega* n − *3 polyunsaturated fatty acid, vitamin and mineral cell complex, neuronal growth, neuroblastoma cells, neurodegeneration*

Introduction

Development and progression of neurodegenerative disease are associated with impaired neuronal growth and reduced neurite length and number (Klein and Ackerman 2003; Zhu et al. 2004). Pharmacological intervention should aim at both of these parameters simultaneously to hamper further development of neurodegeneration and consequent cognitive impairment. Lower Omega *n* − 3 polyunsaturated fatty acid (*n* − 3 PUFA) content has been described in Alzheimer's disease (AD) patients compared to normal age-matched individuals (Conquer et al. 2000; Tully et al. 2003). In elderly subjects, a relationship exists between cognitive decline and decreases in blood *n* − 3 PUFA levels (Heude et al. 2003) and dietary fish intake (Kalmijn et al. 1997, 2004; Kalmijn 2000; Haag 2003). A key role of *n* − 3 PUFA deficiency was shown in a transgenic mouse model of AD (Calon et al. 2004); *n* − 3 PUFAs provide protection from impaired learning ability in AD mice (Moriguchi et al. 2000; Hashimoto et al. 2002) and in normal, aged mice (Carrie et al. 2002). Although it is not clearly established by which mechanism(s) *n* − 3 PUFAs may provide benefit in such situations of cognitive impairment, a role for neuronal growth has been suggested (Ahmad et al. 2002a,b), since *n* − 3 PUFA deficiency leads to decreases in neuronal growth (Ahmad et al. 2002a,b). The *n* − 3 PUFA, docosahexaenoic acid (DHA) promotes neurite outgrowth in cultured hippocampal neurons (Calderon and Kim 2004), and its deficiency leads to decreased neurite length (Calderon and Kim 2004). The protective effects of *n* − 3 PUFA in age-related cognitive impairment may therefore be related, at least in part, to enhancement of neuronal growth.

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Recently, it was observed (Shrivastava and Belani 2003) that certain vitamins, minerals and trace-elements promote cell growth but only when associated in specific proportions (hence cell complex; VMC). Certain vitamins are known to play a key role in neuronal development (Ramakrishna 1999), and the neuronal growth-promoting effects of a number of individual vitamins, including B6 (Morre et al. 1978), B1 and B12 (Fujii et al. 1996), E (Enrione et al. 1999), D3 (Brown et al. 2003) have been reported. Similar effects have also been described for certain trace elements (Tian et al. 2002).

Since antioxidants, cell growth promoting factors and neurite growth enhancers may reduce the pathogenesis of neurodegeneration (Klein and Ackerman 2003), the aim of this study was to investigate the *in vitro* effects of marine-derived $n-3$ PUFAs, eicosapentaenoic acid (C20:5$n-3$; EPA) and DHA (C22:6$n-3$; DHA) as neurite growth enhancers and VMC, as a specific cell growth-promoting complex, alone and in association as a novel investigational approach aiming at reducing *in vitro* neurodegenerative pathology.

Materials and methods

Cell cultures

B 104 rat neuroblastoma cells (ATCC culture collection) were cultured according to the method of Schubert et al. (1974). This cell line was selected because low density seeding differentiates cells into neurons with bipolar neuritis in the absence of serum within 3 days while cell growth is maintained in the presence of serum. To quantify neurite growth and neurite length parameters, cells (approx. 1000/well) were plated onto 48-well microplates while 96-well microplates were used to study growth parameters. Cells were grown on polylysine-precoated microplates using Dulbecco's Modified Eagle tissue culture Medium (DMEM, Gibco, France) containing penicillin (50 IU/ml), streptomycin (50 μg/ml), gentamycin (10 μg/ml) and L-glutamine (2 mM) (all from Eurobio, France). All cultures contained 10% heat-inactivated fetal calf serum (FCS, Eurobio, France) during the first 5–6 h to obtain cell attachment. After this period culture medium was changed with medium containing appropriate concentrations of the test substances with no serum for experiments on neurite growth and 10% FCS for cell growth studies. A serum-free culture medium containing various concentrations of retinoic acid (ATRA) served as positive controls for neurite growth while 10% FCS medium without test substances served as controls for cell growth. Initial experiments were conducted with different concentrations of each test substance to select maximal non-cytotoxic and minimal active concentrations. Stock and experimental cultures were incubated at 37°C in a 90% air—10% CO_2 atmosphere for a period of 3 consecutive days. Each treated culture was accompanied in parallel by an identical but test substance-free control culture.

Test substances

Cell complex. This is a specific association of cell growth promoting vitamins, minerals and trace-elements as described by Shrivastava and Belani (2003); (Naturveda-Vitrobio Laboratories, France, Patent FR 2 838 645-A1). A stock solution containing 650 mg cell complex/ml DMSO was prepared, taking into account its maximum solubility. The initial solution contained (mg/ml): magnesium oxide (338.5), iron gluconate (37.28), ascorbic acid (30.1), beta carotene (20,0), tocopherol acetate (9.0), zinc oxide (7.3), nicotinamide (6.0), pentothenic acid (1.8), pyridoxine hydrochloride (0.67), thiamine hydrochloride (0.47), riboflavin (0.38), folic acid (0.11), biotin (0.07), potassium iodide (0.0654) and inert magnesium stearate stabilizer (198.25). The stock solution was diluted in sterile culture medium (1/50th dilution corresponding to 13 mg/ml cell complex), sedimented (10 min), the supernatant was filtered (22 μm) and used as stock solution. The final concentration selected for further experiments was 1/5th dilution of the stock solution in culture medium, which corresponded to the maximal concentration which produced no cytotoxicity following 120 h exposure to the neuroblastoma cell cultures, as determined in preliminary experiments.

Omega $n-3$ PUFA. Soft capsules of cold water fish skin fat-derived Omega $n-3$ rich oil containing DHA to EPA in a ratio of 1:2.5 (180 mg DHA and 450 mg EPA) were used as a naturally occurring source of marine-derived $n-3$ PUFAs. Cellular bioavailability of PFAs was ensured by mixing PUFA with Bovine serum albumin (BSA, 1.5 mM in 0.15 M NaCl solution) at a ratio of 1:665, which corresponded to the highest soluble concentration of PUFA in BSA without causing serum—oil separation as determined in preliminary experiments. This BSA—PUFA solution was further diluted in serum-free culture medium (1:4) for cell culture exposure, either alone or in association with cell complex solution (1/5th dilution of the stock solution).

Retinoic acid (Sigma, France, R 2625) was used as a positive control for neuronal cultures. An identical BSA solution containing no PUFA was used as a negative BSA control having, in principle, no detectable effect on cellular parameters.

Parameters measured

Cell cultures were exposed to cell complex (VMC), $n-3$ PUFAs or to the association of both for a period

of 3-days. On days 1-3 cell growth was determined using the conventional MTT live cell coloration test. Optical density was measured at 560 nm by means of a microplate reader (Dynatech MR 4000).

Neurite numbers per cell and neurite length were measured with a grilled contrast phase microscope at three different areas in each well for three different wells. Percentage of mean values were calculated at each time point.

Statistical analysis. Comparisons of groups (vs. baseline and vs. vehicle) was performed by one-way ANOVA followed by Student *t*-test *post-hoc*.

Results

Rat neuroblastoma cells in culture were examined for cell numbers, neurite numbers and length on day 0 (before drug or vehicle treatment) and days 1–3 (during drug or vehicle exposure). Four experimental groups were constituted: Group 1: $n-3$ PUFA; Group 2: VMC; Group 3: $n-3$ PUFA combined with VMC and Group 4: Retinoic acid as internal quality control and reference treatment. Cells were examined throughout the study for morphological changes or cytotoxicity.

Cell numbers

In control neuroblastoma cells (no drug treatment), cell numbers ranged from 7.4 ± 0.5 to 9.4 ± 0.6 on day 0 to 14 ± 0.7 to 18.8 ± 1.1 on day 3 ($n = 9$, Table I) in the four groups. In $n-3$ PUFA-treated cells, their numbers increased from 9.0 ± 0.7 on day 0 to 17.4 ± 1.7 on day 3 (P = NS compared to controls). However, in VMC-treated cells, their numbers significantly increased from 8.4 ± 0.3 on day 0 to 23.0 ± 1.6 on day 3 ($p < 0.001$ compared to control group). In $n-3$ PUFA and VMC-treated cells, their numbers were also increased compared to controls, from 8.7 ± 0.5 on day 0 to 20.2 ± 3.2 on day 3 ($p < 0.05$). Retinoic acid had no significant effect on cell numbers compared to the correponding control group (Table I).

Neurite numbers

In control neuroblastoma cells (no drug treatment) neurite number normalized per cell ranged from 0.6 ± 0.1 to 1.2 ± 0.1 on day 0 to 0.4 ± 0.1 to 0.8 ± 0.1 on day 3 ($n = 9$, Table I) in the four groups. In $n-3$ PUFA-treated cells, no significant differences compared to controls were noted, as in cells exposed to VMC (Table I). In $n-3$ PUFA and VMC in association-treated cells, however, a statistically significant increase in the number of neurites occurred over the 3-day treatment period, from 0.8 ± 0.2 on day 1 compared to 0.5 ± 0.1 in controls; 0.9 ± 0.2 on day 2 compared to 0.5 ± 0.1 in controls and 0.7 ± 0.1 on day 3 compared to 0.4 ± 0.1 in controls ($n = 9$, Table I). The effects of retinoic acid on neurite numbers were even more marked, increasing from 0.9 ± 0.5 on day 0 to 1.25 ± 0.06 on day 3 ($p < 0.001$; Table I).

Neurite length

In control cells, neurite length ranged from 0.09 ± 0.01 to 0.12 ± 0.01 on day 0 (no drug exposure) to 0.13 ± 0.01 to 0.15 ± 0.01 on day 3 ($n = 9$, Table I). In $n-3$ PUFA-treated cells, neurite length increased significantly in controls from 0.10 ± 0.01 on day 0 to 0.20 ± 0.02 on day 3 ($p < 0.001$, Table I). However, in VMC-treated cells, no significant effects were noted on neurite length (Table I). In $n-3$ PUFA and VMC-treated cells, significant increases in neurite length occurred, from 0.11 ± 0.01 on day 0 to 0.16 ± 0.02 on day 3 ($p < 0.005$ compared to controls, Table I). In retinoic acid-exposed cells, marked increases in neurite length were noted between day 0 (0.11 ± 0.01) and day 3 (0.30 ± 0.01; $p < 0.001$ compared to controls, Table I).

In summary, treatment of cells with $n-3$ PUFA significantly enhanced neurite length without affecting neurite or cell number; exposure of cells to VMC significantly enhanced cell number without affecting neurite length or numbers; and combined $n-3$ PUFA and VMC-treated cells significantly enhanced all three parameters. Retinoic acid increased neurite length and number, but not cell number.

Discussion

Reduced neuronal regeneration and neurite growth are major factors currently considered to be associated with cognitive impairment caused by neurodegenerative disease. Certain vitamins, minerals and trace elements may reduce the disease symptoms although their mode of action is not yet clearly established. As these elements participate in many cellular reactions as coenzymes or cofactors, or as anti-oxidants, it is likely that they stimulate cellular functions (Ramakrishna 1999; Klein and Ackerman 2003). Several studies have shown that vitamins such as B1, B2, B6, B12 or E improve neuronal function (Ramakrishna 1999). We observed that a highly specific association of these elements (Shrivastava and Belani 2003) is more effective in stimulating cell growth than the individual elements *per se* or when associated in non-specific proportions. Similarly, it has been observed that the $n-3$ PUFAs are very helpful as preventive or curative agents for various neurodegenerative diseases. $n-3$ PUFAs are rich in EPA and DHA, which are integral constituents of cell

Table I. Effect of Omega $n-3$ PUFAs; specific vitamin, mineral and trace element cell complex (VMC); the association of PUFAs and cell complex, and retinoic acid as positive control on the number of cells and neurites, and neurite length determined in neuroblastoma cells *in vitro*.

		Number of cells		No. neurites/cell		Neurite length (mm)	
		CONTROL	TREATED	CONTROL	TREATED	CONTROL	TREATED
Omega-3 PUFA	Day 0	8.9 ± 0.2	9.0 ± 0.7	0.8 ± 0.1	0.8 ± 0.1	0.09 ± 0.01	0.10 ± 0.01
	Day 1	13.1 ± 0.4	13.8 ± 0.7	0.5 ± 0.1	0.7 ± 0.1	0.13 ± 0.01	0.14 ± 0.01
	Day 2	14.9 ± 0.8	15.9 ± 1.0	0.6 ± 0.1	0.6 ± 0.1	0.13 ± 0.01	0.17 ± 0.02
	Day 3	14 ± 0.7	17.4 ± 1.7	0.6 ± 0.1	0.6 ± 0.1	0.13 ± 0.01	0.20 ± 0.02
			p = NS		p = NS		$p < 0.001$
Cell complex (VMC)							
	Day 0	9.4 ± 0.6	8.4 ± 0.3	0.6 ± 0.1	0.8 ± 0.1	0.09 ± 0.01	0.10 ± 0.01
	Day 1	15.3 ± 0.5	12.3 ± 0.6	0.5 ± 0.1	0.6 ± 0.1	0.13 ± 0.01	0.12 ± 0.01
	Day 2	17.7 ± 1.0	17.2 ± 0.8	0.6 ± 0.1	0.4 ± 0.1	0.04 ± 0.01	0.13 ± 0.01
	Day 3	18.8 ± 1.1	23.0 ± 1.6	0.8 ± 0.1	0.5 ± 0.1	0.15 ± 0.01	0.13 ± 0.01
			$p < 0.001$		p = NS		p = NS
Cell complex (VMC) + Omega-3 PUFA							
	Day 0	7.4 ± 0.5	8.7 ± 0.5	1.2 ± 0.1	0.9 ± 0.1	0.12 ± 0.01	0.11 ± 0.01
	Day 1	11 ± 1.1	12.9 ± 1.1	0.5 ± 0.1	0.8 ± 0.2	0.15 ± 0.03	0.18 ± 0.02
	Day 2	12.8 ± 1.2	15.8 ± 2.0	0.5 ± 0.1	0.9 ± 0.2	0.11 ± 0.01	0.18 ± 0.02
	Day 3	16.7 ± 0.5	20.2 ± 3.2	0.4 ± 0.1	0.7 ± 0.1	0.13 ± 0.01	0.16 ± 0.02
			$p < 0.05$		$p < 0.005$		$p < 0.005$
Retinoic acid	Day 0	7.4 ± 0.5	9.0 ± 0.5	1.2 ± 0.1	0.9 ± 0.1	0.12 ± 0.01	0.11 ± 0.01
	Day 1	11.0 ± 1.1	11.7 ± 0.7	0.5 ± 0.1	0.9 ± 0.1	0.15 ± 0.03	0.18 ± 0.01
	Day 2	12.8 ± 1.2	16.1 ± 0.9	0.5 ± 0.1	1.2 ± 0.1	0.11 ± 0.01	0.25 ± 0.01
	Day 3	16.7 ± 0.5	14.8 ± 1.2	0.4 ± 0.1	1.3 ± 0.1	0.13 ± 0.01	0.30 ± 0.01
			p = NS		$p < 0.001$		$p < 0.001$

membranes, and it is likely that their deficiency may be involved in the development and progression of neuronal disorders. Recent studies show that to improve neurodegeneration-related disorders, it is important to stimulate the growth and number of neurites (Zhu et al. 2004) whilst concomitantly stimulating cell growth in the disease-affected tissue. A double approach of stimulating cell regeneration and/or proliferation and at the same time stimulating neurite growth is therefore worthy of consideration. The aim of this study was to investigate for the first time this double approach on neuronal cells *in vitro*.

$n-3$ PUFA deficiency is associated with cognitive decline (Heude et al. 2003), and neuronal growth is impaired (Ahmad et al. 2002a,b), which is amenable to reversal by a $n-3$ PUFA-enriched diet (Kalmijn et al. 1997, 2004; Kalmijn 2000; Haag 2003). The beneficial effects of $n-3$ PUFA in age-related cognitive impairment may be related at least in part to enhancement of neuronal growth (Salem et al. 2001; Ahmad et al. 2002a,b; Catalan et al. 2002; Moriguchi and Salem 2003). However, it is unclear as to how $n-3$ PUFAs exert their effects on neuronal growth parameters. The objective of the present study were therefore twofold: To confirm the growth-enhancing effects of $n-3$ PUFA effects in cultured rat neuroblastoma cells, and to investigate the putative growth-promoting effects of a highly specific association of vitamins and minerals (cell complex; Shrivastava and Belani 2003), based on the established actions of a number of vitamins and certain trace elements (Morre et al. 1978; Fujii et al. 1996; Enrione et al. 1999; Ramakrishna 1999; Tian et al. 2002; Brown et al. 2003), alone and in association with $n-3$ PUFA.

Treatment of neuroblastoma cells for 3 days with $n-3$ PUFA significantly enhanced neurite length without affecting the number of neurites or cells. Exposure of cells to VMC significantly increased cell numbers without affecting neurite length or their numbers; and treatment with $n-3$ PUFA and VMC in a specific association significantly enhanced all three parameters. Retinoic acid, used as a reference treatment, increased neurite length and number, but not cell number, in agreement with previous observations (Simpson et al. 2001). $n-3$ PUFAs are essential for maintaining neuronal function, since their deficiency leads to behavioral and functional deficits (Salem et al. 2001; Ahmad et al. 2002a,b; Murthy et al. 2002; Moriguchi and Salem 2003). Importantly, Ahmad et al. (2002a,b) observed that $n-3$ PUFA deficiency decreased the size of neurons in the hippocampus, hypothalamus and cortex in weanling and mature rats raised on a $n-3$

PUFA-deficient diet, and decreased neuron size is associated with loss of function (Ahmad et al. 2002a,b; Moriguchi and Salem 2003). The data from the present study therefore confirm the neuronal growth-promoting effects of $n-3$ PUFA, and describe growth-enhancing properties in these neurons of a vitamin and mineral complex, in agreement with previous observations (Morre et al. 1978; Fujii et al. 1996; Enrione et al. 1999; Ramakrishna 1999).

The most important observation made in the present investigation is the complementary neuronal growth-enhancing effect of $n-3$ PUFA in a specific combination with VMC. $n-3$ PUFA alone enhanced neurite length, VMC alone increased cell numbers, with neither affecting neurite numbers, whereas in combination, significant enhancement of cell number, neurite length and number occurred. Although the precise mechanism(s) involved remain elusive at present, further *in vivo* studies are warranted to investigate the effects of the combination of $n-3$ PUFA and VMC in models of cognitive impairment.

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