Increase of Neuronal Sprouting and Migration Using 780 nm Laser Phototherapy as Procedure for Cell Therapy

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Background and Objectives: The present study focuses on the effect of 780 nm laser irradiation on the growth of embryonic rat brain cultures embedded in NVR-Gel (cross-linked hyaluronic acid with adhesive molecule laminin and several growth factors). Dissociated neuronal cells were first grown in suspension attached to cylindrical micro-carriers (MCs). The formed floating cell-MC aggregates were subsequently transferred into stationary cultures in gel and then laser treated. The response of neuronal growth following laser irradiation was investigated.
Materials and Methods: Whole brains were dissected from 16 days Sprague–Dawley rat embryos. Cells were mechanically dissociated, using narrow pipettes, and seeded on positively charged cylindrical MCs. After 4–14 days in suspension, the formed floating cell-MC aggregates were seeded as stationary cultures in NVR-Gel. Single cell-MC aggregates were either irradiated with near-infrared 780 nm laser beam for 1, 4, or 7 minutes, or cultured without irradiation. Laser powers were 10, 30, 50, 110, 160, 200, and 250 mW.
Results: 780 nm laser irradiation accelerated fiber sprouting and neuronal cell migration from the aggregates. Furthermore, unlike control cultures, the irradiated cultures (mainly after 1 minute irradiation of 50 mW) were already established after a short time of cultivation. They contained a much higher number of large size neurons ($P<0.01$), which formed dense branched interconnected networks of thick neuronal fibers.
Conclusions: 780 nm laser phototherapy of embryonic rat brain cultures embedded in hyaluronic acid—laminin gel and attached to positively charged cylindrical MCs, stimulated migration and fiber sprouting of neuronal cells aggregates, developed large size neurons with dense branched interconnected network of neuronal fibers and, therefore, can be considered as potential procedure for cell therapy of neuronal injury or disease. Lasers Surg. Med. 41:277–281, 2009. © 2009 Wiley-Liss, Inc.

Key words: axonal sprouting; cross-linked hyaluronic acid with laminin gel; embryonic nerve cells; low power laser irradiation; microcarriers

INTRODUCTION

The therapeutic effect of low power laser irradiation (LPLI) was detected on peripheral and central nervous systems [1–6]. Previous studies, which evaluated the effects of LPLI on crushed injured peripheral nerves of rats, discovered protective immediate effects which increase the functional activity of the injured peripheral nerve [7]; maintenance of functional activity of the injured nerve over time [8]; decrease or prevention of scar tissue formation at the injured site [9]; prevention or decreased degeneration in corresponding motor neurons of the spinal cord [10]; increase in the rate of axon growth and myelinization, thus accelerating and improving the regeneration of the injured nerve. LPLI was found to increase migration and neurite sprouting of cultured embryonic nerve cells [11], as well as cultured adult brain microexplants [12], and to alter gene expression of olfactory ensheathing cells [13]. Our previous studies found that LPLI accelerated axonal growth into injured rat's spinal cord after an implantation of a composite implant, which was based on embryonic spinal cord nerve cells and cultured on biodegradable microcarriers (MCs) that were embedded in hyaluronic acid [6].

In this in vitro study we investigated the effect of 780 nm laser phototherapy on growth and development of embryonic brain neurons and their fibers in culture.

MATERIALS AND METHODS

Cell Culture

Sixteen-day-old rat embryos' (Sprague–Dawley) whole brains were dissected. After mechanical dissociation with narrow pipettes, 5×10^6 cells were suspended in medium attached to DE-53 positively charged cylindrical MCs in 60 mm bacteriological plastic dishes as previously described [14]. After 4–14 days in suspension, the formed floating cell-MC aggregates were collected and seeded in NVR-Gel (in 12 wells or 35 mm plastic dishes) as stationary cultures. Each single cell-MC aggregate was either treated with LPLI within 1 hour after seeding, or cultured without irradiation.

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NVR-Gel

NVR-Gel was produced by NVR^{TM} (Neuro-Vascular Reconstruction Labs, Ness Ziona, Israel). It is composed of crossed linked hyaluronic acid enriched with adhesive molecule laminin (Sigma Chemical Co., St. Louis, MO, USA), 10 ng/ml BDNF (brain developed neuronal factor, Alomone Labs, Jerusalem, Israel) and 4 ng/ml insulin growth factor 1 (IGF-1, Sigma, Life Sciences). The gel is used at a concentration of 0.7% hyaluronic acid and 30 μg/ml laminin [15].

Low Power Laser Irradiation (LPLI)

Irradiation experiments were run in triplicates, at room temperature, using a continuous wave of 780 nm laser beam. The plastic covers were removed from the wells or the culture dishes. The irradiated area was 40–50 mm^2 and the beam angle was 90°. Laser powers were 10, 30, 50, 110, 160, 200, and 250 mW. Each cell-MC aggregate, embedded in NVR-Gel, was irradiated for 1, 4, or 7 minutes.

Culture Medium

Nutrient medium consisted of 90% DMEM-F12 (Gibco® Invitrogen, UK), 10% FCS (Biological Industries), 0.2% antibiotic–antimycotic (Gibco-BRL, Carlsbad, CA), 2 mM glutamine (Gibco-BRL). Glucose was adjusted to 6 g/L.

Fluorescent Staining

Cultures were fixed with 4% paraformaldehyde and incubated with antibodies against neural cell marker: mouse anti-rat microtubule associated protein (MAP-2, Sigma, Life Sciences, diluted 1:30). Cells were then washed and incubated with Texas Red conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The stained cultures were examined under Olympus X70 fluorescent microscope. The number of large neural cells and perikarya size presented in the cultures was detected following 24 days from seeding in NVR-Gel under phase-contrast Olympus X70 microscope and photographed. The pictures in the different treatments were analyzed by comparison neuronal sprouting and perikarya size.

Statistical Analysis

Statistical analysis was performed by Student's t-test. Results are expressed as mean ± standard deviation (SD). $P < 0.05$ was accepted as indicating statistical significance.

RESULTS

Significant differences emerged between control and irradiated cultures. The first difference, consisting of a rapid sprouting of nerve processes from the irradiated cell-MC aggregates, was detected already within 24 hours after seeding (Fig. 1). The extension of nerve fibers was followed by active neuronal migration. Other differences, between controls and irradiated station cultures, became more evident by the end of the first week of cultivation. Already at this early stage, several neurons in the irradiated cultures exhibited large perikarya ($n = 10$), of about 25–30 μm in diameter, and its extending processes 10–100 μm (Fig. 2); irradiated cultures showed thick elongated fibers (Fig. 3). There were no significant differences in perikarya size between control (2.7 ± 0.5), 4 minutes (4.3 ± 1.2) or 7 minutes (3.0 ± 3.0) of 50 mW irradiated cells ($P > 0.05$). However, significant differences were obtained between control (2.7 + 0.5) and 1 minute (8.6+1.15) of 50 mW irradiated cells ($P < 0.01$) (Fig. 4). Furthermore, during the next 2–3 weeks of cultivation, neurons in the irradiated cultures developed dense branched interconnected network of neuronal fibers (Fig. 5). The sprouting of long processes from large cell body was mainly observed in immunofluorescent MAP-2 staining (Fig. 6). In addition, larger areas of ependyma cells were observed in the irradiated cultures. The control cultures unlike irradiated ones contained smaller neurons of about 10–15 μm bearing thin processes. It should be mentioned however, that the beneficial effects were observed during 2–4 weeks in cultures that were irradiated for 1 and 4 minutes but not in cultures which were irradiated for 7 minutes, with the same irradiation intensity. The 7 minutes irradiated cultures showed similar values to the non-irradiated cultures.

The laser irradiation of 50 mW for 1 minute was the most effective and significant of all plethora of powers and time irradiations (10, 30, 50, 110, 160, 200, and 250 mW for 1, 4, or 7 minutes), and had obtained the dual effect of enlarging perikarya size as well as sprouting.

Fig. 1. Effect of laser irradiation on initial sprouting and cell migration from DE-53 Cell-MC aggregates seeded in NVR-Gel. Initial sprouting and cellular migration were observed in irradiated cultures already within 24 hours after seeding in NVR-Gel. **A**: Non-irradiated control. **B**: Single irradiation of 50 mW, for 1 minute. **C**: Single irradiation of 50 mW for 4 minutes. Original magnification: 200×.

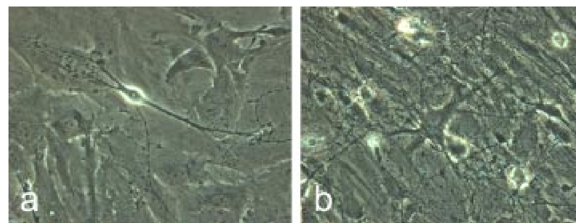

Fig. 2. Effect of laser irradiation on size of nerve somas and nerve fibers of neurons migrated from cell-MC aggregates seeded in NVR-Gel. Neurons in their second week of cultivation: **a**: Non-irradiated control. **b**: After a single irradiation of 50 mW for 1 minute. Original magnification: 200×.

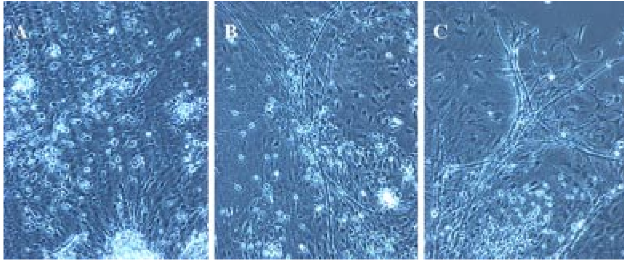

Fig. 3. Effect of laser irradiation on fiber outgrowth from cell-MC aggregates cultured in NVR-Gel. Note branching of thick nerve fibers in (**B**) (4 minutes) and (**C**) (7 minutes) after single irradiation of 50 mW in comparison with (**A**) control. Original magnification: 100×.

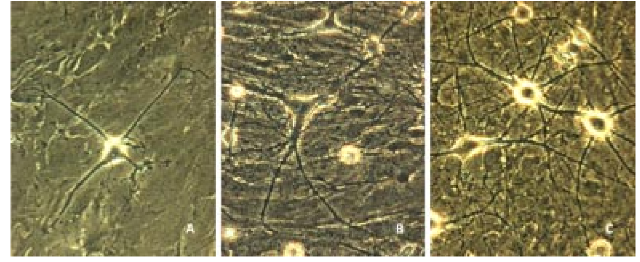

Fig. 5. Differences in nerve cell body size and fiber outgrowth between controls and laser irradiated cultures of rat embryonic brain. A number of large neurons, bearing thick branched neuronal fibers were observed in the irradiated cultures (**B**: 1 minute and **C**: 4 minutes irradiation) as compared to controls (**A**). Original magnification: 300×.

DISCUSSION

The present study focuses on the effect of laser phototherapy on the growth of embryonic rat brain cultures attached to MCs and embedded in hyaluronic acid—laminin gel. Primary embryonic brain cultures are a suitable model for investigating the effect of isolated parameters on culture growth, such as: sprouting, cell and perikarya size. Camu and Henderson [16] that developed method for purifying embryonic rat motoneurons showed also that neuron size reflects neuron physiological and biochemical characterization, since large motoneurons had choline acetyltransferase activities/cell approximately fourfold greater than those of dissociated total spine cells and sevenfold higher than those of the small motoneurons. Sprouting and fiber outgrowth occurs also in rodents and primates, during recovery of sensorimotor function following unilateral cerebrocortical injury or stroke and is accompanied by new neuronal sprouting and synapse formation in adjacent brain regions surrounding focal infarcts and in regions homologous to infarcted cortex in the contralateral hemisphere [17,18]. Moreover, neuronal migrations and beating along glial fibers depend on mPar6 α signaling and the centrosome that acts to coordinate cytoskeletal dynamics [19]. It may be possible that mPar6 α signaling is also involved in laser irradiation induced neuronal sprouting and migration.

In order to get a more accurate evaluation of the irradiation effect on the initial neuronal growth period, we have used a combined suspension and stationary culture technology. In the first stage, cells were grown attached to positively charged MCs in suspension for a

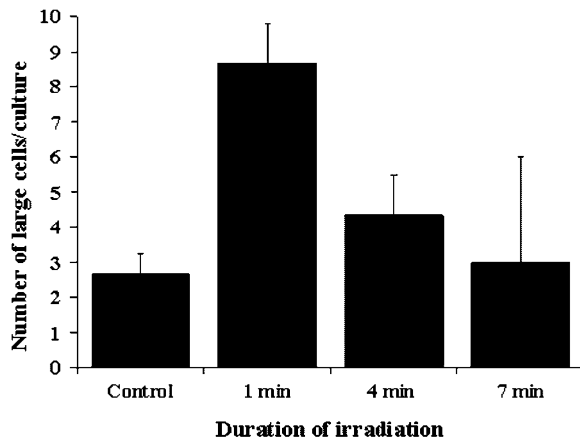

Fig. 4. Effect of laser irradiation on perikarya size of cultured embryonic brain neurons. Cell-MC aggregates were embedded in NVR-Gel after four days in suspension and exposed to a single irradiation of 50 mW. The number of large nerve cells present in the cultures was counted after 24 days using phase-contrast X70 Olympus microscope. Cultures exposed to 1 minute irradiation, contained much larger neurons than cultures irradiated for 4 or 7 minutes as well as non-irradiated control. Data represent the mean ± SD of at least three replicates from a representative experiment ($P < 0.01$).

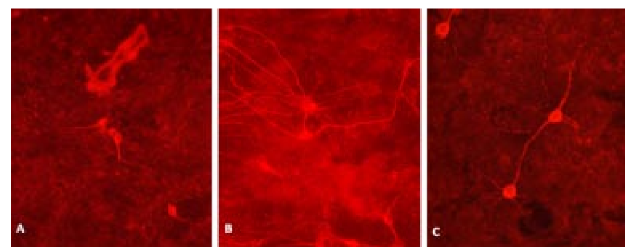

Fig. 6. Immunofluorescent staining of controls and laser irradiated neuronal brain cells migrated from cell-MC aggregates in NVR-Gel. **A**: Non-irradiated control. **B**: 50 mW 1 minute irradiation. **C**: 50 mW 4 minutes irradiation. In the irradiated cultures note large perikarya bearing long interconnected fibers, which are positively stained for the neuronal marker MAP-2. Original magnification: 200×.

few days. Subsequently, the formed floating cell-MC aggregates were seeded in hyaluronic acid—laminin gel as stationary cultures. This way each cell-MC aggregate could be accurately irradiated, unlike the conventional methodology of growing dissociated brain cells, in which a certain area of the monolayer is irradiated.

Laser phototherapy accelerated nerve cell sprouting and cell migration which started already within 24 hours after seeding. Already during the first week of cultivation irradiated cultures contained a high number of neurons exhibiting large perikaria and branched neuronal fibers, which interconnected to form networks. This precocious appearance of large neurons is unlike the usual growth pattern in which neurons grow and become large only after several weeks in culture.

Moreover, the results indicate that not only the intensity of laser irradiation is important but also the duration of irradiation. The importance of time exposure is a very relevant point in future clinical application. To establish the optimal irradiation intensity, we have examined three different durations of irradiation. Our observations showed that the short irradiation durations (1 and 4 minutes) had much better effects on neuronal growth in culture rather than the long exposure of 7 minutes. The long exposure did not induce any noticeable damage to the cultured cells. It is possible, however, that the irradiation was overdosed at this laser intensity. It should be noticed in this connection that lower laser intensities did not have a beneficial effect on neuronal sprouting.

The possible mechanism of action of phototherapy on the nervous tissue with respect to nerve regeneration has been provided by the in vitro studies that showed that phototherapy induced massive neurite sprouting and outgrowth in cultured neuronal cells [11], as well as Schwann cell proliferation [20]. It has also been suggested that phototherapy may enhance recovery of neurons from injury by altering mithochondrial oxidative metabolism [21], and guide neuronal growth cones in vitro, perhaps due to the interaction with cytoplasmic proteins and, particularly, to the enhancement of actin polymerization at the leading axon edge [22]. A possible molecular explanation was provided by demonstrating an increase in growth-associated protein-43 (GAP-43) immunoreactivity in early stages of rat sciatic nerve regeneration after phototherapy [23].

Additional effect of phototherapy on nervous tissue is a neuroprotective action that might facilitate the regenerative process of nerve fibers. It was shown that application of phototherapy upregulates calcitonin gene-related peptide (CGRP) mRNA expression in facial motor nuclei after axotomy. By altering the intensity or temporal pattern of injury-induced CGRP expression, phototherapy may thus optimize the rate of regeneration, target innervation and neuronal survival of axotomized neurons [24]. To sum up, all these functions may play a role in accelerating axonal regeneration and preventing neuronal loss.

The present investigation, as well as the above mentioned studies, support the therapeutic effect of laser phototherapy on nerve cells and can be considered as potential procedure for nerve cell therapy.

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