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RESPONSES OF ASTROCYTES IN CULTURE AFTER LOW DOSE LASER IRRADIATION

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Abstract

The effect of Helium-Neon low dose laser on astrocytes was investigated in cultures of isolated astrocytes from albino neonatal rats. The laser appeared to inhibit the growth of astrocytes as exemplified by the smaller sizes of the cells and the decreased leucine uptake in each cell after treatment. Temporary decrease in the number of mitoses was also observed, but this trend was reversed soon after. Electron microscopic studies revealed an increase in buddings from cell bodies and processes (branches) after irradiation.

KEY WORDS: Laser, Astrocytes, Leucine, Electron Microscopy, Growth, Mitosis, Budding.

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Introduction

Although the laser has gained extensive usage in many clinical areas, its use in the central nervous system is still unexplored. This is largely due to the absence of adequate research in this area and a lot of blanks have to be filled before lasers can be considered applicable to the central nervous system (CNS). In the late 60's, Fox et al. (1967, 1968) and Hayes et al. (1967) in a series of experiments claimed that irradiation with a high dose laser of total energy of 50-100 Joules onto the skulls of animals would lead to brainstem herniations in these animals as a result of intracranial explosion subsequent to the absorption of the intense energy. Many years later, Yew and Chan (1977) in a study of irradiating the central nervous system of animals with a much lower dosage of 7 to 10 Joules of total energy, reported the loss of spines in neurons. Following the introduction of a new hypothesis (Yew et al., 1982b) on the stimulating effect of low dose lasers (lasers generating total energies of less than 1 Joule) on the production of amino acids and mucopolysaccharide in the retina, a lot of attention was subsequently given to the low dose laser effects on the biological systems. These results were further substantiated by the demonstration of laser stimulation of physiological activities in the abdominal ganglion of mollusc (Fork, 1971) and the laser stimulation of functional activities in cultured mouse cells (Berki et al., 1988). In our laboratory, we were interested in how the low dose laser would affect the different major cellular components i.e. glia cells and neurons of the nervous system. This is a report to summarize some of the responses of the low dose laser on the astrocytes which were isolated and cultured.

Materials and Methods

20 neonatal albino rats (Sprague-Dawley) of 2 days age were employed in the experiment. They were sacrificed by decapitation and the cerebral cortices, cerebella and brainstems were dissected under aseptic conditions and meshed in Hank's solution. The suspensions were centrifuged at 1200 rpm for 2 minutes and the supernatants

discarded. The residues were resuspended again in Hank's solution, centrifuged and the supernatants discarded. The latter residues were suspended in Minimum Essential Medium (MEM) culture medium and cultured in flasks. The cell number was 2.5 x 10^5/ml. Fetal calf serum 10 ml/100 ml, penicillin G (Sigma) 100 U/ml, streptomycin sulfate (Sigma) 100 µg/ml and Fungizone (Squibb) 2.5 µg/ml were added to the MEM (Gibco cat 410-1100). The cultured cells were allowed to grow for 7-8 days in the incubator. After that, the primary cultures were removed by 0.05% Trypsin (Sigma type III) and 0.02% Ethylenedinitrilo-tetraacetic acid (EDTA) in Hank's solution. After 2 minutes, an equal volume of culture medium was added and the mixture centrifuged at 1200 rpm. The supernatant was discarded and new MEM medium added. The suspension was reinoculated on either cover slips or collagen plates to form monolayer cultures.

After 3 days of growth, 96 cultures were utilized in further experiments. Half of them were irradiated with a Helium-Neon laser (CW laser, 632.8 nm, Spectra Physics, U.S.A.) of 1 mW power through an optic fiber for 5, 10 and 15 minutes, respectively. The irradiated area in the culture was 12.5 cm^2 in each case. The total energies of irradiation were calculated to be 11 mJ/cm^2, 22 mJ/cm^2 and 33 mJ/cm^2, respectively. The other half of the cultures were left untreated as control specimens. 2 days after irradiation, both control and experimental cultures were employed. Each control or experimental group was divided into four subgroups to be used for glial fibrillary acid protein immunohistochemistry (GFAP), scanning and transmission electron microscopy, labelled leucine uptake and counting of mitoses, respectively. In order to determine whether the effects obtained were indeed from laser treatment, another control group (designated "sham control") was instituted for the subgroup of scanning and transmission electron microscopy. In this sham control group, 6 cultures were used and they were irradiated by monochromatic (632.5 nm) red light with a total energy of 1.4 J/cm^2 for 15 minutes.

For GFAP histochemistry, 3 control and 9 laser treated cultures were used. Out of the 9 laser treated cultures, 3 had 5 minutes of laser treatment 2 days before. 3 had 10 minutes of laser treatment 2 days before and 3 had 15 minutes of laser treatment 2 days before. The cultures (both control and experimental) were fixed in neutral buffered 10% formalin for 24 hours and washed in distilled water, followed by washing in phosphate buffer at pH 7.4. Afterwards, the monolayers were incubated in dilute normal goat serum for 20 minutes followed by incubation for 30 minutes with rabbit antibodies against GFAP (1:300 dilution) (primary antibody) in phosphate buffer. The specimens were then washed and incubated with goat anti-rabbit biotinylated antibody solution (secondary antibody) for 30 minutes. Specimens were washed again for 10 minutes in buffer and reincubated with Vectastain ABC reagent (Vector Lab., California, USA) for 60 minutes. After washing in buffer for 10 minutes subsequently, they were developed in 0.1% diaminobenzidine (DAB) in 0.1 M buffer mixed with an equal volume of 0.02% H_2O_2 for 10 minutes. The specimens were then washed in tap water for 5 minutes, cleared and mounted. Observations were performed with a Nikon microscope. Apart from routine observations, the surface areas of 110 cells taken randomly from the 3 control cultures and 9 laser treated cultures were measured from micrographs and the means and standard deviations were computed.

For scanning and transmission electron microscopy, 6 control, 6 sham control (light treated) and 18 laser treated cultures were used. Out of the 18 laser treated cultures, 6 were treated with laser for 5 minutes 2 days before, 6 were treated with laser for 10 minutes 2 days before and the remaining 6 were treated with laser for 15 minutes 2 days before. For scanning electron microscopy, 3 control, 3 sham control and 9 treated cultures (3 from each laser treated group) were fixed with 2.5% glutaraldehyde in 200 mOsm cacodylate buffer for 2 hours and washed in the same buffer. After washing and dehydration through graded alcohol applications, they were then put into 3 changes of Freon TF of 15 minutes each, followed by critical point drying and coating with gold. Observations were done with a 35 CF JEM scanning electron microscope. For transmission electron microscopy, all 3 control, 3 sham control and 9 treated specimens (3 from each laser treated group) were fixed in 2.5% glutaraldehyde (4°C) in 200 mOsm cacodylate buffer at pH 7.4 for 2 hours. They were then washed in 200 mOsm cacodylate buffer and postfixed for 1 hour in 1% OsO_4 in the same buffer. The specimens were then dehydrated through graded alcohol applications and embedded in Spurr's resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and observed with a JEOL 100CX transmission electron microscope.

For labelled leucine uptake, 3 control and 9 laser treated specimens were used. Out of the 9 laser treated specimens, 3 were treated with laser for 5 minutes 2 days before, 3 were treated with laser for 10 minutes 2 days before and the remaining 3 were treated with laser for 15 minutes 2 days before. Both the control and irradiated specimens were incubated in ^3H-leucine in Hank's solution (1 µ Ci/ml) at 37°C with 95% air and 5% CO_2. After 30 minutes of incubation, they were fixed in 10% neutral buffered formalin and the monolayers were coated with Kodak NTB2 emulsion (Rochester, USA) and left in the refrigerator at 4°C for two weeks. They were then developed in D 19, fixed and washed and the specimens were stained with Hematoxylin and Eosin and mounted. The occurence of labelled granules per astrocyte in each control or experimental group was counted as follows: n = 56 (control), n = 64 (laser treated for 5 minutes), n = 60 (laser treated for 10 minutes) and n = 62 (laser treated for 15 minutes). The means and standard deviations were computed and tabulated.

For mitotic counts, 12 control and 36 experimental specimens were used. Out of the 36 experimental cultures, 12 were irradiated with laser for 5 minutes 2 days before. Another 12 were irradiated with laser for 10 minutes 2 days before and the remaining 12 were irradiated with laser for 15 minutes 2 days before. Colchicine (Sigma) in the concentration of 160 µg/100 ml was

introduced into the culture, and both control and experimental cultures (3 cultures from each group) were obtained after 3, 12, 24, 36 hours of colchicine introduction, respectively. The specimens were then fixed in 10% neutral buffered formalin, stained with Giemsa, dehydrated through graded alcohol applications and mounted. The percentages of mitosis were counted in 10 random areas of 100 cells in each group of culture at each timing and the means and standard deviations computed.

Results

GFAP immunohistochemistry showed that over 90% of the cells in culture were GFAP positive. In the control cultures, there were few cells that assumed star shapes and there were few GFAP positive fibers inside the cells (Fig. 1). After laser treatments, more cells assumed star shapes and more positive GFAP fibers were spotted in each cell (Figs. 2-4). Morphometrical measurements on cell areas revealed that the control cells were 230.6 ± 73.3 μm^2. The cells that had 5 minutes of laser treatment were of sizes 249.6 ± 96.3 μm^2. The cells that had laser treatment for 10 minutes were of sizes 170 ± 33.3 μm^2 and the cells that had laser treatment for 15 minutes were of sizes 152.3 ± 59.6 μm^2. There was thus a gradual decrease in cell

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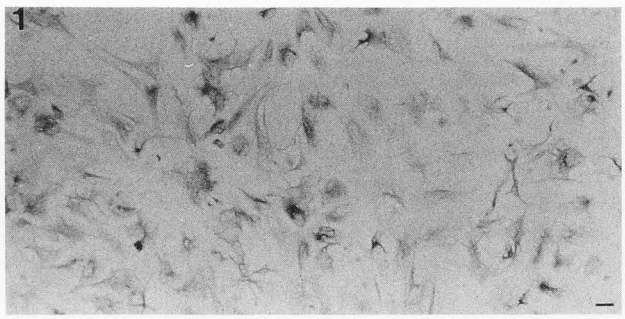
Fig. 1. A normal culture of astrocytes showing GFAP positivity. Bar = 100 μm.

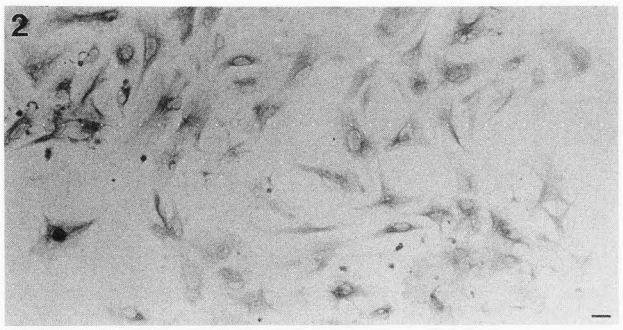
Fig. 2. Laser treated culture of astrocytes (5 minutes) with more GFAP positive fibers when compared with control. Note more cells were star shaped. Bar = 100 μm.

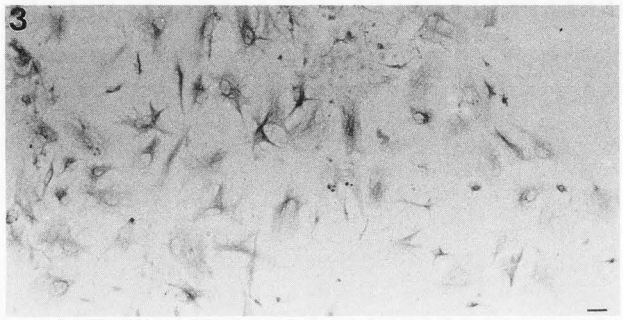
Fig. 3. Laser treated culture of astrocytes (10 minutes) (GFAP immunohistochemistry) with many star shaped cells which were smaller in size. Bar = 100 μm.

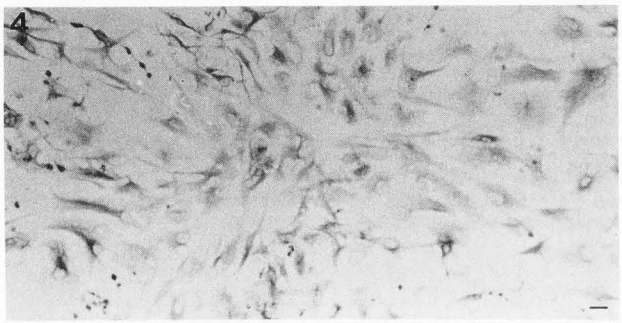
Fig. 4. Laser treated culture of astrocytes (15 minutes) (GFAP immunohistochemistry) showing many cells that were star shaped and smaller in size. Bar = 100 μm.

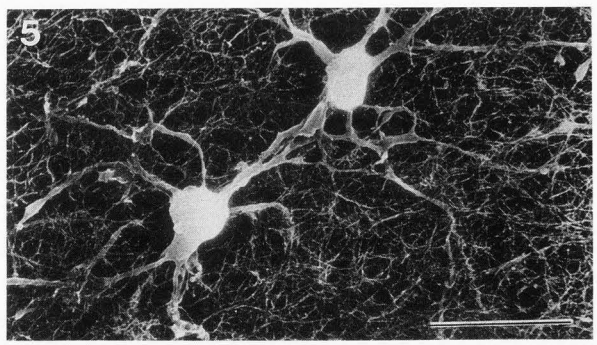
Fig. 5 & 6. Scanning electron micrographs showing 2 types of normal astrocytes in culture. One with oval cell body and long branches (Fig. 5) and the other with round body and short branches, some of which had dilated ends (arrow) (Fig. 6). Bar = 10 μm for both figures. Background fibers were collagen.

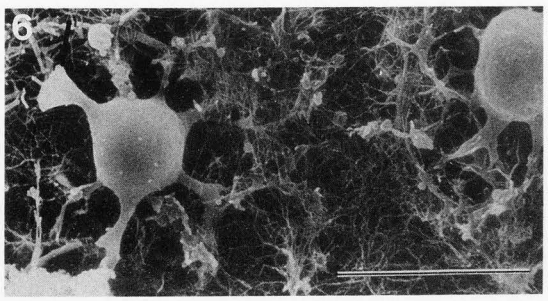












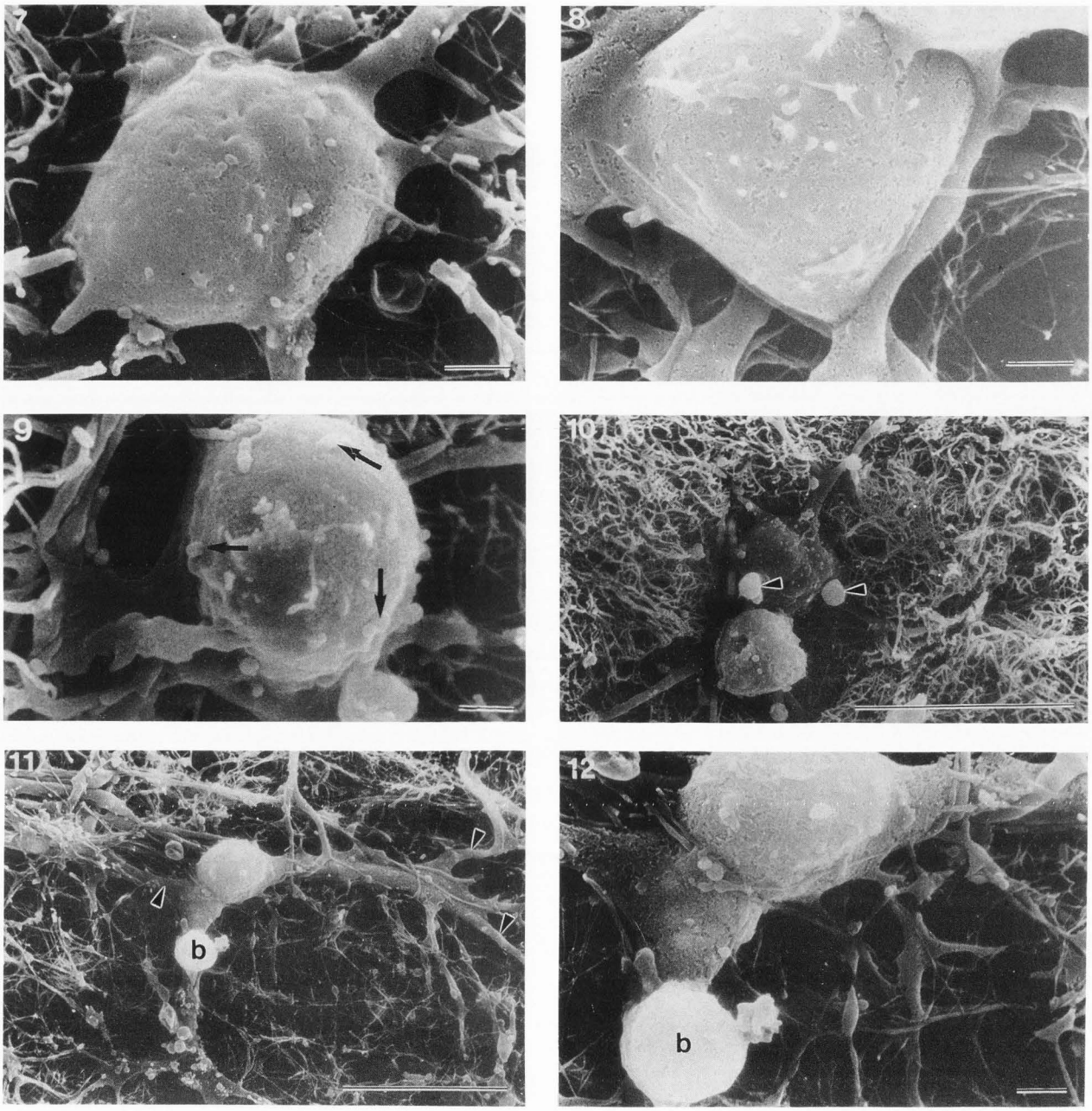


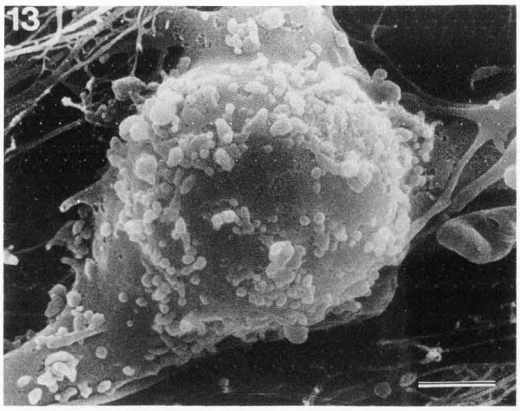
Fig. 7. Scanning electron microscopy showing cell body of control astrocyte with very few surface projections which were usually of a small size. Bar = 1 μm.

Fig. 8. Scanning electron micrograph of sham control astrocyte showing uneven surface but without obvious budding. Bar = 1 μm.

Fig. 9. Scanning electron micrograph showing buddings (arrows) from surface of cell body of astrocyte that had laser treatment for 5 minutes. Bar = 1 μm.

Fig. 10. Scanning electron micrograph of astrocyte in culture that had laser treatment for 10 minutes. Note larger buddings (projections) (arrows) from surface of cell body. Bar = 10 μm. Background fibers were collagen.

Fig. 11 & 12. Scanning electron micrograph of astrocytes that had laser treatment for 10 minutes showing fewer branches (arrows) and a budding (b) (Figs. 11 & 12) from one of the branches in higher power. Bar = 10 μm in Fig. 11 and bar = 1 μm in Fig. 12. Background fibers were collagen.



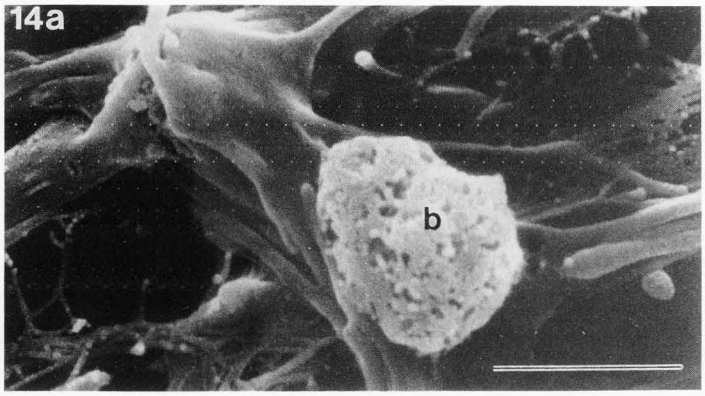


Fig. 13. Scanning electron micrograph showing drastic increase of surface projections (buddings) from the cell body of an astrocyte in culture that had laser treatment for 15 minutes. Bar = 1 μm.

Fig. 14a,b. Scanning electron micrograph of a branch of the astrocyte that had laser treatment for 15 minutes. Note buddings (b or B) from branches. One budding had denuded surface (Fig. 14a). Bar = 1 μm.

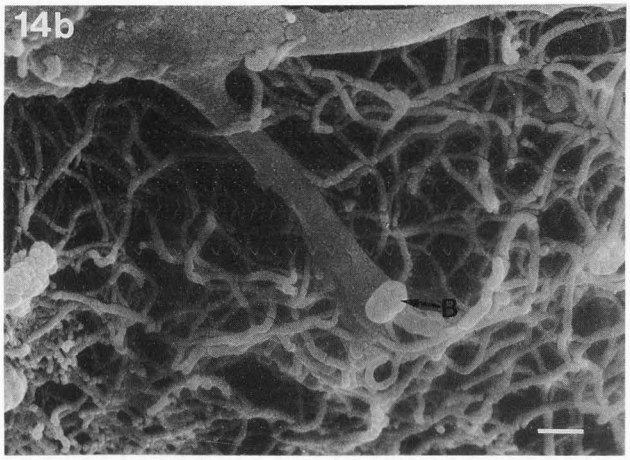
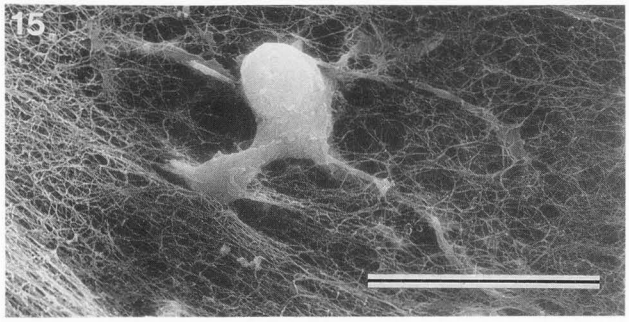
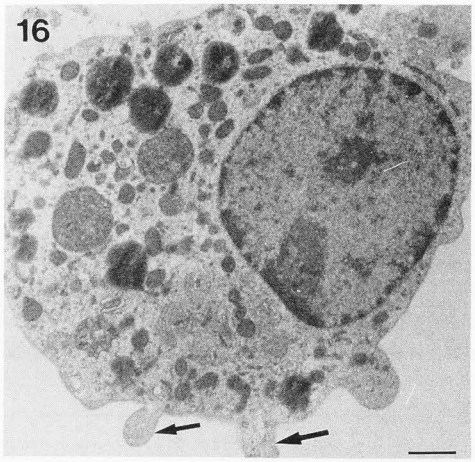


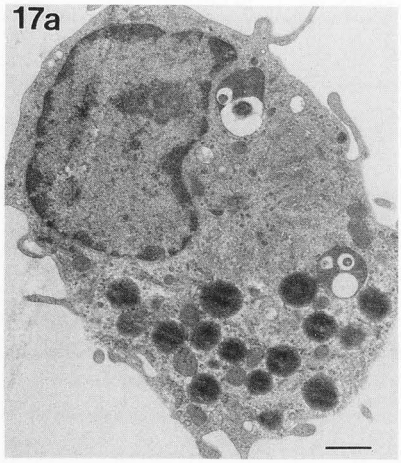
Fig. 15. Scanning electron micrograph of an astrocyte in culture that had laser treatment for 15 minutes. This is an astrocyte that had short branches. Note that there are fewer branches than control and also the branches were of uneven diameters. However , there was no obvious increase of surface projections. Background fibers were collagen. Bar = 10 μm.

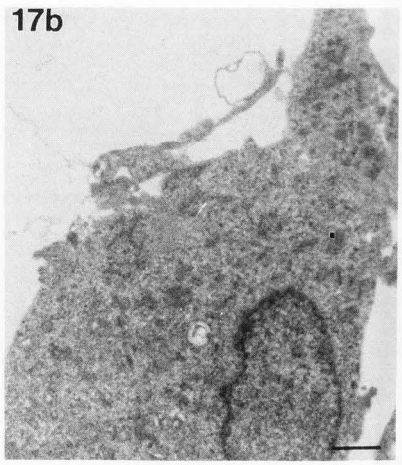


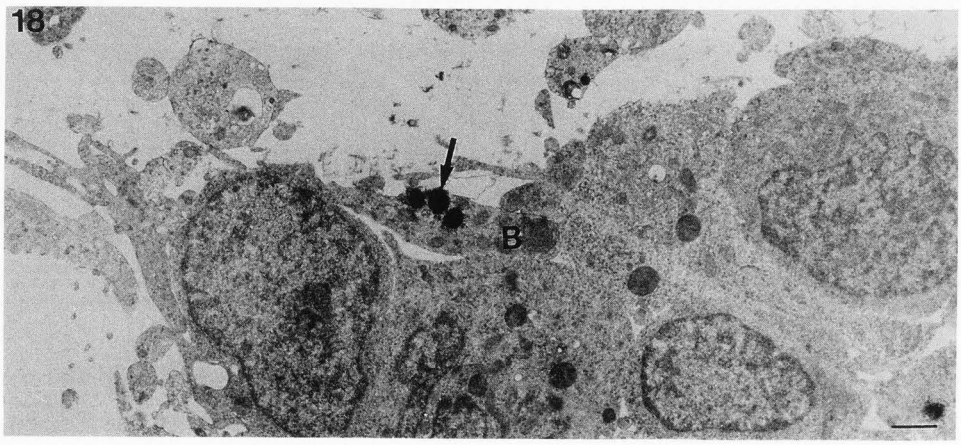
areas (sizes) after laser treatment (t test, $p < 0.001$ in the cells treated with laser for 10-15 minutes when compared with control).

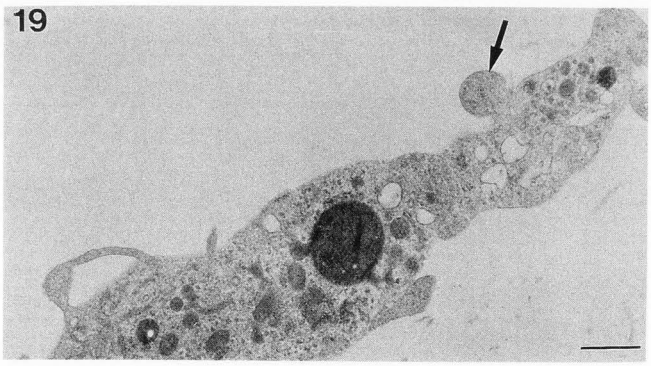
Scanning electron microscopy demonstrated two types of astrocytes in culture. One of these had an oval cell body with long thin projections all round the body (Fig. 5). The other type had a rounded body with shorter projections, also arising from all round the body (Fig. 6). Sometimes, the projections of the latter would form dilated tips (Fig. 6). The majority of the cell surfaces of control and sham control groups were rather smooth, occasionally with a few very tiny round projections no more than 0.2 um in diameters (Figs. 7 & 8). There were only slight size differences between the astrocytes from different areas of the central nervous system. For the sake of clarity, comparisons were only made between the control and experimental astrocytes of the same area so as to avoid complications. In cultures that had 5 minutes of laser irradiation 2 days before, increase of surface projections (buddings) were obvious (Fig. 9). A rough estimation of surface projections per normal control cell and per laser treated cell (5 minutes of treatment) revealed 2.6 ± 0.7 [x (mean) $\pm$S.E. (standard error)] projections in the former group and 4.5 ± 0.64 ($x \pm$S.E.) projections in the latter group. "(probability) $p=0.05$ and $n=100$ cells." In cultures that had laser irradiation for 10 minutes 2 days before, small ball-like projections (buddings) were observed in some astrocytes and these were all of larger diameters than control and averaging around 1.5 μm (Fig. 10). Laser treated astrocytes also had fewer branches (Fig. 11). Ball-like projections were also evident on the branches of some astrocytes and these were very large (e.g. 4 um in diameter) (Fig. 12). In the cultures that had 15 minutes of laser treatment 2 days before, very significant increase in surface projections arising from the cell bodies were noticed and they ranged from diameters of 0.2 to 0.3 μm (Fig. 13). Projections (buddings) from branches of the astrocytes (Fig. 14a, b) were also observed and these projections sometimes had denuded surfaces (Fig. 14a). For the astrocytes with shorter branches, presence of fewer branches were also featured (Fig. 15)

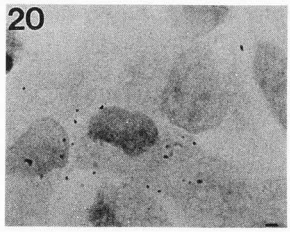












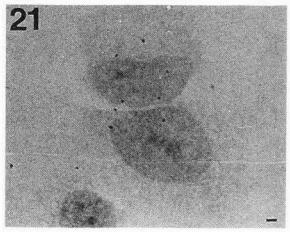


Fig. 16 & 17a,b. Transmission electron microscopy showing an astrocyte that had laser treatment for 15 minutes revealing increased buddings (with no specialized organelles) of larger sizes from the surface (Fig. 16, arrows) when compared with the control (Fig. 17a) and sham control (Fig. 17b). Also note the increase of dense bodies in the cell body of laser treated astrocyte (Fig. 16). Bar = 1 μm in Fig. 16 and Fig. 17a & b.

Fig. 18. Transmission electron micrograph of an elongated protrusion from the surface of astrocyte, that had laser treatment for 10 minutes, contains dense bodies (arrow) inside budding (B). Bar = 1 μm.

Fig. 19. Transmission electron micrograph of buddings (arrow) from branch of astrocyte that had laser treatment for 10 minutes. Note no specialized organelles inside budding. Bar = 1 μm.

Fig. 20 & 21. Autoradiograph of labelled leucine uptake in control astrocyte (Fig. 20) and laser treated astrocyte for 15 minutes (Fig. 21). Note less uptake in laser treated. Bar = 1 μm for both figures.

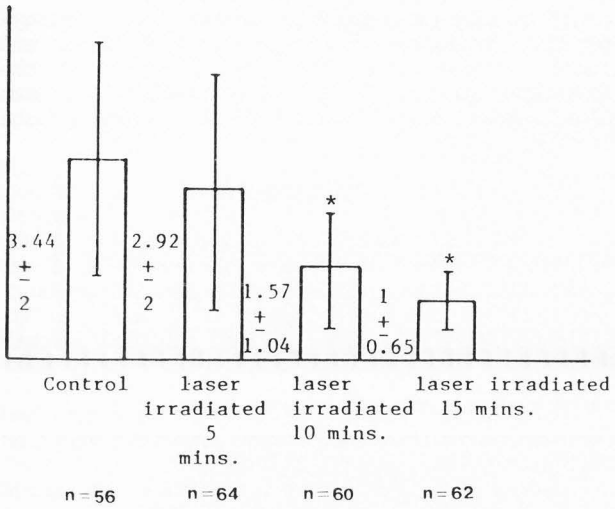


Fig. 22. Histogram of labelled leucine uptake in the astrocytes of control and laser treated. ⊢⊣ denotes standard deviations. * indicates $p < 0.001$ (t test) when compared with control. n = number of astrocytes.

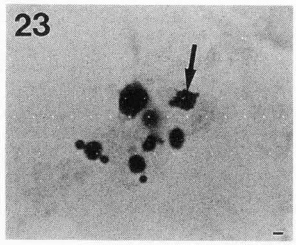


Fig. 23. Light microscopy showing an astrocyte in mitosis (arrow) in the culture environment. Bar = 1 um.

although no increase in surface projection was obvious (Fig. 15). Rough counting indicated an averaged decrease of 20% in the number of branches. Transmission electron microscopy revealed that the increase in the surface projections were evident in the cell bodies after laser treatment, when compared with the control and sham control groups (Fig. 16 & 17a & b). Furthermore, the surface projections of the laser treated cells were in general of larger sizes than the control groups. The surface projections/protrusions after laser treatment might not contain any specialized organelles (Fig. 16) or they might contain dense bodies (Fig. 18) as well as ribosomes. With higher cumulative laser dosage (longer durations of irradiation), buddings arising from the branches of the astrocytes were also evident and these contained no specialized organelles (Fig. 19). Increase in dense bodies of larger sizes inside the experimental astrocytes was evident when compared with control, after laser treatment (Fig. 16 & 17).

Labelled leucine uptake indicated a continual decrease in the leucine uptake in the astrocytes subsequent to laser treatment (Fig. 20, 21 & 22), $p < 0.001$ (t test) in specimens treated with laser for 10 & 15 minutes.

Figure 23 shows mitosis in an astrocyte and mitotic counting indicated that subsequent to laser irradiation, there was a possible decrease of mitosis 24 hours after application of colchicine (i.e. 3 days after irradiation) (Fig. 24); $p < 0.01$ & $p < 0.001$ (t test) in different groups of different treatment durations when compared with the control. However, this trend was reversed after another 12 hours (36 hours after colchicine application) (Fig. 24); $p < 0.001$ (t test) in all treated groups when compared with the control group.

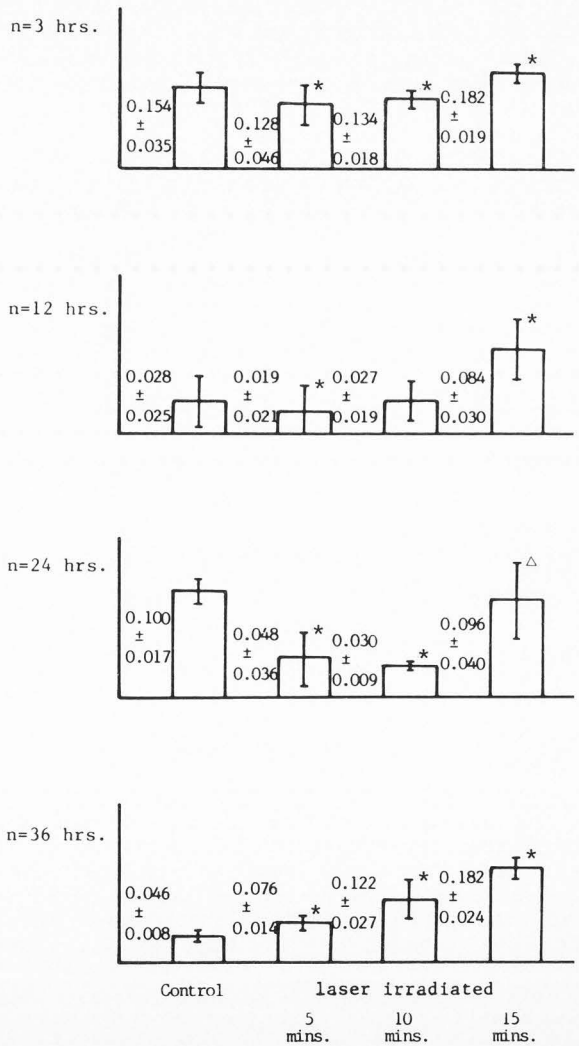


Fig. 24. Histogram of the percentage of mitosis in the populations of astrocytes in control and laser treated cultures. n = hours after colchicine application. ⊢⊣ denotes standard deviation. * indicates $p < 0.001$ (t test) when compared with control. Δ indicates $p < 0.01$ (t test) when compared with control.

Discussion

This work pointed out that laser treatment could in fact affect astrocytes. Inhibition of growth of these cells was indicated by the smaller sizes (surface areas) of cells in the culture and the decreased amounts of leucine uptake by the cells after laser treatment. On the other hand, this decrease in size (surface area) could well be the result of degeneration with contraction and rounding up partially and might not reflect real changes in volumes. The decreased amount of leucine uptake might suggest decreased protein metabolism associated with retarded growth. Inhibition of growth of cells in cultures after laser irradiation has also been documented for pigment cell cultures (Yew et al., 1982a). However, inhibition of growth did not necessarily mean that the cells were less mature. In fact, our GFAP immunohistochemistry proposed that there appeared to be more positively reactive fibers after laser treatment in the smaller cells, thus hinting that the laser treated cells might be more differentiated or more mature. Furthermore, although from our results, there appeared to be a decline of mitoses a few days after laser treatment, such decline was rapidly balanced off by an increased burst of mitoses in the short period that followed. The inhibiting effect of the laser on mitoses was therefore questionable. Increased thymidine incorporation into the pigment cells in culture after low dose laser irradiation was reported by our group (Tsang et al., 1986). If this earlier result is applicable for all types of cells in culture, it will mean that low dose laser not only will not inhibit mitoses, but tends to enhance it.

Subcellular changes in the astrocytes after low dose laser treatment were typified by increased surface projections (buddings). This increase was unique for the laser as our sham control (irradiated with light of the same wavelength and with higher energy) did not produce any obvious changes similar to those of the laser treatment. This was different from earlier reports that monochromatic or polarized light treatment produced membrane changes (Boder et al., 1983; Kubasova et al., 1988) or fluorescent light reduced colony diameter and altered cell morphology in cultured cells (Bradley & Shakey, 1977). The difference was obviously due to the laser wavelength and the type of cell used. The increase in budding initially appeared in the cell body after laser irradiation and then later in the branches, after irradiation with a higher dose. It is tempting therefore to suggest perhaps that the branches of the glial cells are more radioresistent and react to higher dosages of radiation. The "budding" (or blebbing) is a well known pathological phenomenon and has been documented in respiratory and urinary system after laser irradiation by our group (Mok et al., 1988). Furthermore, there seemed also to be an increase in the quantity of dense bodies inside the cells after irradiation and these bodies were secondary lysosomes of the residual body variety indicating perhaps cell damage with resulting autophagolysis.

Low dose laser, unlike high dose laser, does not emit a high intensity of light which after being transformed into the enormous heat energy, results in the microexplosions of the cells and causes severe damage and scarring. Many of the low dose laser effects will eventually be much more useful clinically and therefore warrants more studies.

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Discussions with Reviewers

Z. Somosy: What is the definition of "low dose" of laser irradiation?
Authors: In our laboratory, we define "low dose" laser irradiations as those of total energies below 1J.

Z. Somosy: To what extent are the experimental changes specific for laser light?
Authors: From the results gathered by our group

employing the He-Ne laser, we felt that the changes were probably specific for laser light as our "sham control" which had been irradiated with normal light, of the same wavelength, and with higher energy did not reveal the same changes.

M. Albertsson: Could you discuss more the mechanisms of buddings? Degradation phenomenon? Heating effects?
Authors: We felt that the mechanisms of budding were probably related to the changes in the cytoskeleton of the cells after irradiation. Cyclic AMP, adenyl cyclase as well as Ca^{++} might be involved. We did not think this was heat related as the heat effect was very minimal at this very low dosage.

M. Albertsson: Why do dense bodies increase after laser treatment?
Authors: The dense bodies were probably lysosomes indicating perhaps cell damage. The increase in quantity of dense bodies might also point to an increased rate of cellular catabolism or degradation.

U. Brunk: Why was that pure gold had been used for metal coating in SEM instead of Au/Pd or Pt? Furthermore, fixation was done at a hypo-osmotic pressure.
Authors: Pure gold coating is also an acceptable way of metal coating although Au/Pd is probably more superior in view of its particle sizes. However, within the present magnifications used in this study, the difference is not so critical. Fixation was done in buffer of close to 226 mOsm, according to Fahimi and Drochmans (J. Microscopie 4, 737, 1965) and has worked well for our samples in our laboratory for many years.

T.D. Allen: The dense bodies in Fig. 18 do not occur in blebs, and therefore cannot be compared with a true bleb in Fig. 19.
Authors: The term "bleb" or "budding" in our opinion refers to pathological surface projection which may or may not be round and regular. The bleb in Fig. 18 is irregular and is a bit compressed in contour. Nevertheless, this is a pathological surface projection which according to definition is a true bleb or true budding.