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CONTRACTILITY CHANGES IN CULTURED CARDIAC CELLS FOLLOWING LASER MICROIRRADIATION OF MYOFIBRILS AND THE CELL SURFACE

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SUMMARY

Laser irradiation of myofibrils in cultured neonatal rat heart cells at a wavelength of 537 nm produced subsarcomeric lesions that were visible in the phase contrast, polarizing and electron microscopes. This irradiation resulted in the production of contractile changes as frequently as the microbeaming of mitochondria. Irradiation of the cell surface via carbon particles produced similar contractile changes more often than either myofibrillar or mitochondrial irradiation. The contractile changes noted in this study appear to be dependent on extracellular calcium.

Contractility in living cells, its subcellular basis and its control are presently areas of intense research. The application of lasers to these investigations has been a relatively recent development [1–3]. Laser microirradiation of individual mitochondria in cultured myocardial cells can produce lesions as small as $0.25\ \mu\text{m}$ and induce contractile responses which can be correlated with the morphology of the damaged organelle [4]. Light and electron microscopy have confirmed the localization of the damage to the target mitochondrion but have provided no specific information concerning the relationship between mitochondrial lesions and changes in cell contractility.

The first direct evidence of the involvement of the cell membrane in the contractile response to mitochondrial irradiation came from the work of Kitzes et al. [5]. They showed by intracellular electrophysiological recordings from microirradiated

myocardial cells that membrane depolarization accompanied mitochondrial microbeaming and the contractile response. The present studies were undertaken to further probe this relationship.

We report here the following. (1) It is possible to specifically irradiate myofibrils located just beneath the cell surface and produce subsarcomeric lesions that are visible in the polarizing microscope and the electron microscope. (2) Myofibrillar lesions result in contractility changes as often as do mitochondrial lesions. (3) Arrhythmic contractions result from myofibrillar lesions to either the A band or the I-Z region. (4) Irradiation of the myocardial cell surface by the direct microbeaming of adhering carbon particles results in altered contractile responses in single cells and groups at a much lower irradiation energy and with a much greater frequency than either mitochondrial or myofibrillar irradiation. (5) This contrac-

tile response is dependent on extracellular calcium. (6) A similar contractile response cannot be elicited by microbeaming the myofibrils or the cell surface of spontaneously twitching cultured skeletal muscle myotubes.

MATERIALS AND METHODS

Primary cultures of neonatal (1–3 days old) rat ventricular cells were established in Rose chambers as described in Waymire et al. [6]. The original inoculum was between 3×10^5 and 5×10^5 cells per chamber. Non-muscle cells were allowed to attach to the coverslip on one side of the chamber during the first 2 h of culture (preplating) and then the Rose chamber was turned over to allow the bulk of the muscle cells to attach on the other side. Cultures were examined each day using phase contrast microscopy and were fed every 2–3 days beginning on day 3. Large myofibrils were first seen in the phase microscope in flattened myocardial cells on the 4th or 5th day of culture and became larger and more obvious as the culture period proceeded. Irradiations were usually performed on day 6 or 7.

Chick embryo skeletal muscle cultures were prepared as described by Bischoff & Holtzer [7]. 3×10^5 cells were inoculated into Rose chambers prepared with collagen-coated glass coverslips. Cultures were fed daily and myotubes were present on day 4. Spontaneous twitching activity was apparent by day 7 or 8, and irradiations were performed at this time.

Irradiation was accomplished using a Q-switched Neodymium-YAG laser focused by a modified phase contrast microscope in a set-up essentially similar to that described by Berns [8] and Rattner et al. [4]. The primary difference is replacement of the argon laser by the more powerful Nd-YAG system. Wavelengths of 532 and 537 nm were used since they are close to the visible light absorption maximum of myoglobin, a naturally occurring chromophore in these cells [9]. Laser output at 532 nm was 5.9 kW with a pulse duration of 100–150 nsec; at 537 nm, output was 2.0 kW with a pulse duration of 100–150 nsec. Calibrated neutral density filters were used to attenuate laser output energy to produce mitochondrial lesions of weak to moderate severity [4, 10]. This system allows the precise aiming of the laser microbeam via the alignment of the target structure with a pre-aligned crosshair on a television monitor and the simultaneous observation and videotaping of the entire irradiation process. Only beating cells, whether isolated or in small groups, were irradiated.

Contractile responses to laser microbeaming were monitored for several minutes following irradiation, and several were recorded on videotape for later analysis. Irradiated cells were assayed for myofibrillar lesions by polarizing microscopy using a modified Zeiss RA microscope equipped with a quartz-halogen lamp and Planachromat objectives. 0.5 mM EGTA in Ca-Mg-free Hanks' solution or heart medium without

added calcium was used to prevent cells from contracting during photographing.

Cells destined for electron microscopy were fixed in situ following irradiation in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2–7.4. After post-fixation in buffered 1% osmium tetroxide and en bloc staining in aqueous uranyl acetate, the cultures were dehydrated in ethanol and embedded in Epon 812 in the normal manner. Glass coverslips were removed from the embedded cultures by immersion in liquid nitrogen, and the irradiated cell was relocated, marked and cemented to a blank Epon block for serial thin sectioning. Sections were collected on slotted formvar coated grids, stained in uranyl acetate and lead citrate, and examined on a Siemens Elmiskop 1A or a JEM 100C at 80 kV.

For experiments involving the irradiation of the cell surface, carbon particles were used as the chromophores. A stock solution of 4% Aquadag (particle size 1–2.5 μm) was prepared in sterile Hanks' solution containing 10 times the normal medium concentration of penicillin and streptomycin and was allowed to stand overnight. This was then diluted 1:100 with sterile heart medium and injected into the culture chambers. Particles were allowed to attach to the cells for several hours, and unadhering particles were removed prior to irradiation by gentle washing with fresh medium.

RESULTS

Morphology of lesions

More laser energy was generally required to produce myofibrillar lesions than mitochondrial lesions [see 4, 10]. Two or three 100–150 nsec pulses at 537 nm were required to produce obvious mitochondrial damage while five or six pulses were needed to produce even subtle changes in a sarcomere that could be observed with the phase contrast microscope (figs 1, 2). At least 50 mitochondria and 50 myofibrils were irradiated in these experiments. Both A and I-Z band irradiations were made. In both

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Fig. 1. Neonatal rat cardiac muscle cell (a) in culture for 7 days. Note prominent myofibrils. Arrow, sarcomere to be irradiated; (b) immediately following laser irradiation. Arrow indicates lesion in A band. $\times 2160$.
Fig. 2. Neonatal rat cardiac muscle cell (a) in culture. Note large mitochondria. Arrow indicates mitochondria to be irradiated; (b) immediately following laser irradiation. Arrow indicates pale irradiated mitochondrion with phase dense center. Adjacent mitochondria appear unaffected. $\times 2160$.

Fig. 3. Polarizing microscope pictures of myofibril in cardiac cell in culture. A bands are bright. Arrow indicates sarcomere (a) prior to irradiation; (b) imme-

diately following laser irradiation with a wavelength of 537 nm.

cases, the immediate effect seemed to be a slight transitory increase in the length of the irradiated sarcomere, but this could not be confirmed photographically due to the required exposure times and the contractile activity of the cells.

Polarizing microscopy was used to confirm the presence of myofibrillar lesions. Loss of birefringence from irradiated anisotropic bands (A bands) was pronounced even when damage could not be seen using conventional phase contrast microscopy. One example is shown in fig. 3. Lesions ranged from subtle palings of the A band about 1 μm in diameter to the almost complete loss of birefringence of the target area. The smaller the number of laser pulses

given, the smaller and less dramatic the lesion. The polarizing microscope was less helpful in identifying lesions in the region of the I and Z bands. Damage to this region could often be inferred by the increase in the distance between adjacent A bands. Direct evidence that lesions could be made in the I-Z band area was obtained by electron microscopy, but finding the laser damage in fixed and sectioned material was often difficult. This difficulty could be overcome, however, by irradiating a large mitochondrion close to the target myofibril. The mitochondrial lesions produced in these studies were similar to those described as "weak" or "moderate" by Adkisson et al. [10] and Rattner et al. [4]. In the phase con-

Fig. 4. Electron micrograph of myofibril with laser-induced lesion in A band. Adjacent sarcomeres appear normal. $\times 30\,000$.

Fig. 5. Electron micrograph of cardiac muscle cell following irradiation of Z band. Note expanded Z band while adjacent sarcomeres appear normal. $\times 13\,300$.

trast microscope, the mitochondria paled leaving one or two phase dense spots near the center of the organelle (fig. 2). These mitochondria were also readily identifiable in the electron microscope and were used to mark the position of the myofibrillar

lesions. Figs 4 and 5 illustrate representative A band and I-Z band lesions as seen in the electron microscope. Discrete damaged areas about $0.5\text{--}1\ \mu\text{m}$ in diameter could usually be seen in A bands, and thick and thin myofilaments appeared equally af-

Table 1. *Contractile responses to laser irradiation*

Irradiated organelle	Medium	λ (nm)	No. of pulses	Final laser output (kW) ^b	Contractile response	
					no.	%
Myofibrils	NHM	537	3	0.74	18/50 ^c	36
A bands	NHM	537	3	0.74	8/25	32
I-Z bands	NHM	537	3	0.74	7/25	28
Mitochondria	NHM	537	3	0.74	16/50	32
Cell surface (Carbon particles)	NHM	532	1-2	0.003	49/50	98
Mitochondria	NCA+1 mM EGTA	532	3	1.18	0/8	0
Mitochondria	NCA+0.5 mM EGTA	532	3	0.05	0/11	0
Cell surface (Carbon particles)	NCA+0.5 mM EGTA	532	3	0.000046 ^a	0/11	0
	NCA+0.5 mM EGTA→NHM	532	3	0.000046 ^a	9/10	90
Mitochondria	NCA+0.5 mM EGTA→NHM	532	3	0.05	4/10	40

NHM, normal heart medium; NCA, heart medium without added calcium; EGTA, ethyleneglycol-*bis*-tetraacetic acid; →, medium changed.

^a Note low energy at which cell surface irradiation of carbon particles induces a contractile response.

^b None of the powers listed below should be considered thresholds for the observed effects.

^c In this experiment, no attempt was made to selectively irradiate subsarcomeric regions whereas A bands or I-Z regions were selectively irradiated in the experiments below.

fects. At the center of the lesion, filaments were obscured by electron-dense material, but they were seen at the periphery. In every case, sarcomeres adjacent to the irradiated sarcomere appeared completely normal.

Lesions to the I-Z area (fig. 5) had a totally different character. It appeared that the dense material which composed the Z band had expanded laterally and unevenly toward adjacent sarcomeres. The damage could be detected in 6-8 serial sections to varying degrees and appeared to be spherical.

Contractility changes

Laser irradiation of myofibrils at 537 nm produced changes in myocyte contractility similar to those described as a "fibrillation response" following mitochondrial irradiation [4, 6, 11, 12]. Contractions became rapid and irregular, and this pattern often lasted for several minutes. Such contractility changes occurred even when great care was taken to avoid irradiation of the mitochondria lying adjacent to the target

myofibrils. In several cases, cells that contracted irregularly following myofibrillar irradiation were examined ultrastructurally, and no obvious mitochondrial damage was found outside the mitochondrion that was intentionally marked. Since mitochondrial marking was always done following myofibrillar irradiation, it could not have caused the contractility change.

Less laser energy was required to produce changes in myocyte contractility than to make an obvious myofibrillar lesion (2-3 pulses vs 5-6) as detected with polarizing or electron microscopy. This same amount of energy (2-3 pulses) was sufficient to make weak or moderate mitochondrial lesions. Thirty-six percent of myofibrillar irradiations and 32% of mitochondrial irradiations resulted in contractility changes of the type described. It thus appears that myofibrillar and mitochondrial lesions are equally likely to result in contractility changes. We attempted to determine whether I-Z band or A band-centered myofibrillar irradiations were more likely to result in arrhythmic contractions. Rapid asynchronous contrac-

Fig. 6. Beating cell from neonatal rat heart culture with carbon particles adhering to cell surface. Particles are 1–2.5 μm in diameter and are attached singly or in groups (arrows). $\times 1100$.

tions resulted from 7/25 I-Z band irradiations and 8/25 A band irradiations, approx. 30% in each case (see table 1).

In order to test whether laser irradiation of the myocardial cell surface would cause a contractile response, carbon particles 1–2.5 μm in size were used as chromophores. These particles were attached to the cell surface as described in Materials and Methods (fig. 6). In one set of experiments, irradiation of the adhering particles resulted in arrhythmic contractions lasting several minutes in 49/50 cells. Only 1/600 of the energy required to make mitochondrial lesions that are visible in the phase contrast microscope was necessary to trigger this response. Mitochondrial irradiations at the

same low energy resulted in a contractile response in only two of eleven cases, and both of these cells resumed their pre-irradiation beating rates within 30 sec.

To determine whether extracellular calcium was necessary for the altered contractile states induced by laser irradiation, beating heart cells were placed in heart medium containing no added calcium plus EGTA. Cells immediately ceased their spontaneous rhythmic activity in this medium. No contractile responses were elicited following mitochondrial, myofibrillar or carbon particle irradiation at either 1 mM or 0.5 mM EGTA. When EGTA-containing medium was replaced with normal medium (2.5 mM calcium), spontaneous beating activity resumed. Irradiation of cell surface carbon particles and mitochondria in beating cells from these same chambers now elicited the expected contractile responses. A summary of these results is presented in table 1.

Several attempts were made to induce contraction of skeletal muscle myotubes via laser microbeaming. Neither myofibrillar nor mitochondrial irradiation produced a contractile response. Wavelengths of 532 nm and 537 nm were used, but despite the ability of myotubes to twitch spontaneously in culture, contractions were never produced.

DISCUSSION

In this paper we report and document the feasibility of using the Nd-YAG laser microirradiating system in investigations of cardiac myofibrils. Lesions of 0.25–1.0 μm in diameter can be made in specific sarcomere regions and can be identified with polarizing and electron microscopy. No artificial chromophore is required, and advantage can be taken of visible light absorption by myoglobin. We are presently using

this system to ask questions concerning intracellular regeneration and repair in cardiac cells. In addition, this system now makes it possible to investigate various systems of cytoplasmic filaments other than myofibrils. Succeeding publications will deal extensively with microfilaments and filaments of intermediate diameter.

Control of contractility in cultured cardiac cells has been the subject of numerous recent articles and reviews [13, 17, 18], and several authors have suggested that the calcium ion plays a major role in this regulation. The ability of cardiac mitochondria and sarcoplasmic reticulum (SR) to sequester calcium has been documented [14, 18], but the precise level of free calcium required to trigger a contractile response in heart cells is still unknown [18]. It has been speculated that laser irradiation of mitochondria causes calcium to be liberated into the cytoplasm where it could result in a temporary change in contractility [4], but up to now, little direct evidence was available to confirm or deny this hypothesis. The irradiation of myofibrils in the present studies has been shown to result in contractile changes as frequently as mitochondrial irradiation. Troponin, the calcium binding protein present in myofibrils, appears to have a lower calcium affinity than either mitochondria or SR [18], and it is difficult to assess whether enough could be liberated from a single irradiated sarcomere to trigger the responses found in the present work. The location of the myofibrils in cultured cardiac cells close to the cell membrane and the close structural relationship between myofibrils and the sarcoplasmic reticulum make it almost impossible to irradiate myofibrils without affecting both of these structures. Thus, mitochondria, SR, the cell surface, the external medium and the myofibrils themselves are all possible

sources of the calcium required for the laser-induced contractile response. Introduction of calcium from any of these sources could create a situation similar to that which has been proposed to account for the altered contractile patterns found following mitochondrial irradiation.

The results presented here emphasize the importance of the cell membrane to the contractile response. The irradiation of carbon particles adhering to the cell surface resulted in contractile changes at 1/600 of the energy required to produce such changes by irradiating either mitochondria or myofibrils. Uncoordinated, arrhythmic contractions were induced even when the target carbon particles were situated over the cell nucleus. Salet et al. [12] found that UV irradiation of the nucleus with a wavelength of 254 nm was relatively ineffective in producing a contractile response despite the fact that this wavelength is absorbed by virtually all biological molecules including those of the cell membrane. However, the energy density used in those studies was relatively low compared with those used in the present work. Given this low energy density and the physical distance which separated the irradiated nucleus from the contractile apparatus of the cell, it is interesting that several cells did contract arrhythmically following nuclear irradiation in Salet's studies.

The importance of the cell membrane in the observed contractile effects has also received considerable support from the electrophysiological data obtained by Kitzes et al. [5]. These workers found that mitochondrial microirradiation with an argon laser resulted in membrane depolarization which accompanied a change in contractile pattern. Especially interesting in that report is the distinction between the behavior of pacemaker and non-pacemaker

cells in response to the laser. It is likely that both pacemaker and non-pacemaker cells were among the 49 cells which showed asynchronous contractions following cell surface microbeaming via carbon particles in the present work. By visual criteria, all reacted similarly. Our inability to distinguish between these two cell types might have been due to the different wavelengths and energy produced by the argon laser and the YAG system. To our knowledge, no data are presently available on how carbon particles attached to the cell surface transfer and distribute the energy which they absorb. If pacemaker and non-pacemaker cells reacted similarly in the present studies, the results obtained emphasize their similarity rather than their differences.

Additional support for the role of the cell membrane in the observed laser-induced contractile effects come from our observations concerning EGTA. We never succeeded in inducing contractions of any kind when mitochondria or surface-attached carbon particles were irradiated in medium with no added Ca^{2+} in the presence of this calcium chelator. Based on the similarity of the calcium binding constants of EGTA, mitochondria and SR [14, 15], it is likely that considerable intracellular calcium remained despite the presence of EGTA in the external medium. This implies that the laser induced a contractile response via its effect on the cell membrane, causing an increase in permeability to Ca^{2+} resulting in a Ca^{2+} influx.

The capacity of laser irradiation to cause changes in contractile activity in heart cells can be contrasted with its inability to cause similar changes in cultured skeletal muscle.

This difference is not surprising based on the well known physiological differences between skeletal and cardiac muscle [16]. Though myofibrillar irradiation leads to contraction in heart cells, the myofibrils themselves are probably the effectors rather than the initiators of the response. It will be interesting to determine what, if any, contractile effects laser irradiation has on smooth muscle and the contractile elements of non-muscle cells.

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