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LASER MICROIRRADIATION OF STRESS FIBERS AND INTERMEDIATE FILAMENTS IN NON-MUSCLE CELLS FROM CULTURED RAT HEART

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SUMMARY

Stress fibers and bands of intermediate filaments (100 Å) were studied in cultured non-muscle cells using laser microbeam techniques. Wavelengths of 532, 537 and 280 nm were used, and no artificial chromophores were employed. Lesions were assayed using a combination of phase contrast, polarizing and transmission electron microscopy (TEM). (1) Stress fibers 1–2 µm in diameter were narrowed or completely severed by irradiation at 532, 537 and 280 nm. Stress fibers could be grouped into two classes: (a) those whose severed ends separated during the first few seconds following laser irradiation (46% of fibers irradiated); (b) those fibers which showed no movements (54%). Microtubules which paralleled stress fibers persisted in the presence of colcemid for up to 5 h, and alignment of the severed stress fiber ends was maintained even in their absence. Injured stress fibers appear to be repaired within 1 h of irradiation. (2) Bands of 100 Å filaments were induced in non-muscle cells in secondary cultures of neonatal rat heart by exposure to colcemid. Lesions which appeared as phase dense spots were induced in these bands by irradiation at 532, 537 and 280 nm. The positions of the lesions in the band relative to one another did not change over several hours despite movements of the entire band. These studies demonstrate that (a) stress fibers may be an excellent system in which to study subcellular repair; (b) induced bands of 100 Å filaments probably move passively in the cells containing them; (c) laser irradiation of cytoplasmic filaments in non-muscle cells does not require the introduction of an artificial chromophore.

We recently reported the specific laser microirradiation of myofibrils in cultured neonatal rat heart cells [1]. In those studies, we used a neodymium-YAG laser at a wavelength of 537 nm, close to the absorption maximum of the soluble muscle pigment, myoglobin [2]. We showed that it was possible to induce contractile changes in the irradiated cells and make subsarcomeric lesions in either the A bands or I-Z band region. The extent and specificity of those lesions could be assessed using a combination of phase contrast, polarizing and electron microscopy.

We report here the specific laser micro-

irradiation of two additional systems of cytoplasmic filaments in non-muscle cells derived from cultured rat heart, namely "stress fibers", and bands of filaments of intermediate diameter. These experiments differ from those reported previously in three basic respects: (1) The cells irradiated in these studies are non-muscle cells obtained by subculturing primary cultures of neonatal rat heart; (2) these cells contain little, if any, myoglobin, a naturally occurring chromophore in heart muscle cells; (3) laser wavelengths in the green range of the visible spectrum (532 and 537 nm) were used in these studies. No naturally oc-

curing chromophore with absorption in this range is known to exist in these cells, and no artificial chromophore was added. Despite the apparent lack of a chromophore, lesions visible in the phase contrast, polarizing and electron microscope were produced in stress fibers and bands of filaments of intermediate diameter. The lesions induced by laser irradiation at 532 and 537 nm were compared with lesions induced by UV laser irradiation at 280 nm. The results of irradiations at the three wavelengths were similar. The use of laser irradiation has enabled us to make several observations concerning cytoplasmic filaments which correlate well with their previously determined biochemical nature.

MATERIALS AND METHODS

Neonatal rat ventricular cells were grown in Rose chambers as described in Waymire et al. [3]. Cells were plated at a density between 3×10^5 and 5×10^5 per chamber and were fed every 2–3 days beginning on day 3. These cultures contained between 40–50% beating heart myocytes, and the remainder were non-muscle cells. The growing cells were detached from the substrate after 7–10 days via direct injection of 0.125% Viokase-EDTA into the chambers. Following resuspension in fresh medium, cells were seeded into fresh chambers at 5×10^5 cells/chamber. Secondary cultures contained less than 1% beating cells and were allowed to grow for 3–5 days before subculturing again. Tertiary cultures were plated in Rose chambers at 3×10^5 cells/chamber and consisted of broad, extremely flat, non-muscle cells with extensive stress fibers. Stress fiber irradiations were always performed on the 2nd or 3rd day following the establishment of the tertiary cultures. Cells were grown on either glass or quartz coverslips depending upon whether visible or UV laser wavelengths were used. Quartz coverslips were obtained from Esco Products, Oak Ridge, N.J., and were 0.32 ± 0.05 mm in thickness.

Bands of 100 Å filaments were induced in secondary cultures of neonatal rat heart by incubation in medium containing 10^{-6} M colcemid. Colcemid-containing medium was added on day 2 of secondary culture and phase lucent birefringent bands became evident in approx. 40–50% of the cells within 48 h [4, 5]. Irradiation of bands of 100 Å filaments was done 48–72 h following the addition of colcemid in the presence of colcemid-containing medium.

Cells were irradiated at 532 or 537 nm wavelengths using a Q-switched, neodymium-YAG laser focused by a modified phase contrast microscope described by

Strahs et al. [1]. 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 lesions in the target structures of the desired severity. (The lesions had to be severe enough to be easily seen in the phase contrast microscope while not killing the cell). UV irradiation at a wavelength of 280 nm was accomplished by combining the Nd-YAG laser with an organic dye laser as described in Berns et al. [6]. Laser output at 280 nm was 200–500 W with a pulse duration of 80 nsec. UV attenuating filters were used to adjust laser intensity to produce lesions in stress fibers and bands of 100 Å filaments similar to those produced by laser irradiation in the visible range. A 100× Neofluar phase objective was used to focus the laser at 532 and 537 nm while a 32× quartz Ultrafluor phase objective was used at 280 nm. UV-induced lesions were compared with lesions produced at visible wavelengths.

Immediately following laser irradiation and phase contrast photography, cells were examined by polarizing microscopy using a modified Zeiss RA microscope with planachromat objectives and a Xenon light source (Zeiss XB075). Cells were photographed using Tri-X film and exposure time was about 1 min.

Cells were prepared for TEM as described by Strahs et al. [1]. Following fixation in situ in 3% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2, cells were washed in buffer, post-fixed in 1% osmium tetroxide and stained en bloc in saturated aqueous uranyl acetate. Cultures were dehydrated in ethanol and embedded in Epon 812 in the normal manner. Glass or quartz 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 JEM 100C operated at 80 kV.

Cell extracts to be used for spectrophotometry and gel electrophoresis were prepared as follows. For each experiment, 4–6 whole hearts were removed from 2 day old rats and were placed in cold Hanks' BSS with out phenol red. They were dissected free of all visible adhering connective tissue and were washed in three changes of cold BSS. The extraction procedure was similar to that used by Reynafarje [7]. Hearts were weighed, homogenized in 0.05 M Tris-HCl buffer, pH 7.5, in a glass tissue homogenizer in the cold and were allowed to stand on ice for 10 min. The extract was transferred to a centrifuge tube and spun at 12 000 g for 12 min at 4°C. The pellet was discarded and aliquots of the supernatant were filtered through a Millipore (0.4 µm) and examined for absorbance in the 400–600 nm range on a Beckman ACTA III recording spectrophotometer. Horse heart myoglobin to be used as a standard was obtained from Sigma Chemical Co. and was dissolved at 0.1 mg/ml in 0.05 M Tris-HCl, pH 7.5. Protein content of the soluble extracts was determined by the method of Lowry et al. [8] and 10% polyacrylamide slab gels were run according to the method of Studier [9]. Gels were stained for 3–5 h in Coomassie Brilliant Blue and destained overnight.

Extracts of tertiary heart cultures were prepared as

Fig. 1. (a) Phase contrast micrograph of the cytoplasm of a non-muscle cell from a tertiary heart culture. Note numerous stress fibers (arrows). (b) Same cell as (a) after microirradiation of a single stress fiber indicated

by the arrow. Irradiation was performed at a wavelength of 532 nm. No movement was seen in the irradiated fiber or the adjacent cytoplasm. $\times 2080$.

above except that cultures were grown to confluency in 75 cm² T-flasks. Cells were detached from the substrate with a rubber policeman.

RESULTS

Stress fiber irradiations

Figs 1 and 2 show lesions in stress fibers in cells of tertiary heart cultures immediately following laser irradiation at wavelengths of 532 nm and 537 nm. Fig. 3 shows a similar lesion immediately following UV irradiation at a wavelength of 280 nm. The results of the irradiations at the three wavelengths were similar.

The number of laser pulses required to produce the type of lesions shown in figs

1–3 varied with the size of the target stress fiber. Narrow fibers about 1 μm in thickness usually required 3–4 pulses at 532 nm and 5–6 pulses at 537 nm. Two or three pulses at 280 nm were usually sufficient to sever a fiber of this diameter. The responses of the target stress fibers to irradiation at any of these wavelengths could be divided into two categories. In approx. 54% (15/28) of the cases, the distance between the cut ends of the irradiated fibers (0.25–0.50 μm at 100 $\times$ and 1–2 μm at 32 $\times$) remained constant following irradiation. No movement was noted in the fiber or the adjacent cytoplasm, and the precise alignment of the cut ends was maintained.

In the remaining 46% (13/28) of the stress

Fig. 2. (a) Phase contrast micrograph of a non-muscle cell from a tertiary heart culture. Note large stress fiber (arrow); *(b)* same cell as *(a)* after microirradiation of stress fiber at wavelength of 537 nm. Lesion is indicated by arrow. Cut ends of fiber moved apart for several seconds after irradiation. $\times 2080$.

fiber irradiations, the severing of the target fiber was immediately followed by the separation of cut ends. Within 30 sec to 1 min, the gap between the cut ends of the fiber expanded to a distance several times the size of the initial lesion (example shown in fig. 2). Several irradiations were performed in the presence of 0.5 mM EGTA in Ca-Mg-free Hanks' solution or in 0.5 mM EGTA in heart medium without added calcium. Despite the presence of this calcium chelator, five out of eleven fibers retracted. Alignment of the fiber was always maintained, but it was impossible to determine whether or not the cut ends increased in thickness.

Evidence from polarizing microscopy following laser irradiation of stress fibers sup-

ported the severing of the fibers that was seen in the phase microscope. However, in several cases, fibers thought to be completely cut by virtue of the phase contrast image were seen to be merely narrowed or attenuated when viewed in polarized light. Such an example is shown in fig. 4. Complete severing of a fiber was not necessary for fiber movement, and numerous instances of movement were noted following stress fiber narrowing.

*Fig. 3. Phase contrast micrographs of non-muscle cell from tertiary heart culture (a) before; (b) after UV laser microirradiation of stress fiber at wavelength of 280 nm. Lesions is indicated by arrow. $\times 525$; (c) electron micrograph of a section of same cell as *(a, b)* showing lesion in stress fiber. Lesions is indicated by arrow. $\times 6000$.*

Fig. 6. Phase contrast micrographs of non-muscle cell from tertiary heart culture following 5 h of incubation in medium containing 10^{-6} M colcemid. (a) Stress fiber prior to laser microirradiation at a wavelength of 532 nm; (b) 30 min following laser microirradiation of stress fiber at two points along its length indicated by arrows. The irradiated fiber appears to be completely severed at one point but narrowed at the other. Note that alignment of the segment between the two lesions has been maintained. $\times 1700$.

Fig. 8. Phase contrast micrographs of non-muscle cell from heart subculture following 48 h incubation in 10^{-6} M colcemid. (a) Phase lucent band composed of filaments of intermediate diameter is indicated by arrow; (b) same cell following laser microirradiation of band of filaments at three points (arrows) at wavelength of 537 nm. $\times 3200$.

Fig. 4. Polarizing microscope picture of cultured cell showing numerous birefringent stress fibers. Arrow indicates loss of birefringence from area of stress fiber which has been laser irradiated at wavelength of 532 nm. Fiber does not appear to be severed. $\times 900$.

Fig. 5. Electron micrograph of non-muscle cell from tertiary culture of neonatal rat heart. Cell has been incubated for 1 h in medium containing 10^{-6} M colcemid. Note microtubule (mt) running parallel to stress fiber (SF). $\times 30000$.

Fig. 7. "Healing" of stress fiber in cardiac non-muscle cell. (a) Preirradiation; (b) immediately following irradiation at 532 nm, 5.9 kW/pulse, 100–180 nsec/pulse. Arrow indicates lesion. Note central phase dense spot; (c) 1 h after irradiation. Stress fiber is once again complete. Diminished central spot marks site of lesion (arrow). $\times 1660$.

Several instances of laser-induced severing and narrowing of stress fiber bundles were also confirmed by TEM of serial thin sections. An especially interesting result of these experiments was the consistent finding of small numbers of microtubules running parallel to the stress fibers. Some of these microtubules appeared intact following laser irradiation of a nearby stress fiber, and this led to the hypothesis that intact microtubules were responsible for maintaining the alignment of the ends of an interrupted fiber. To test this hypothesis, colcemid was added to medium in which tertiary heart cultures were growing. Cells from these cultures were examined by electron microscopy. Following 1 h of exposure to 10^{-6} M colcemid, the only microtubules which remained intact were those which paralleled stress fibers (see fig. 5). These microtubules were absent, however, following 5 h of exposure to colcemid. Irradiations of stress fibers at wavelengths of 280 and 532 nm were performed in the presence of colcemid following 5–6 h of pre-incubation in the drug. Two types of experiments using colcemid were performed. The first type involved the irradiation of a fiber at 532 nm at one point along its length, and the cell was observed continuously for 1 h via videotape. Six cells were in this group. In the second type of experiment (9 cells), stress fibers were irradiated at 532 nm at two points along their length such that an "isolated" segment at least 1–2 μm in length was produced (fig. 6). These cells were also observed for 1 h following irradiation. In 14 of 15 of these experiments, including both the above groups, the alignment of the stress fibers was maintained following severing and retraction in the absence of cytoplasmic microtubules. In the one case where some lateral movement of the cut stress fiber was noted, the cell died within

10 min of irradiation. Similar results were obtained following irradiation at 280 nm.

In approx. 90% of stress fibers irradiated, cut ends of the fiber had rejoined within 30–45 min of irradiation (fig. 7). A phase dense spot often marked the original site of the lesion. "Healing" appeared to take place even when retraction of the completely severed fibers resulted in a discontinuity of several micrometers in length, and it also occurred in the presence of 10^{-6} M colcemid. This phenomenon has been noted following irradiation at 532 nm, 537 nm and UV irradiation at 280 nm. Further experiments are presently underway to identify the nature of and the requirements for this subcellular repair.

Irradiation of bands of intermediate filaments

Approx. 40–50% of cells in secondary and tertiary heart cultures developed phase lucent birefringent bands following 24–48 h exposure to 10^{-6} M colcemid [4, 5]. These bands have been shown to consist of tightly packed and oriented filaments of about 100 Å in diameter. As demonstrated previously [10], the bands of filaments move extensively with time while the cells containing them are immobile. Laser lesions were made in bands of intermediate filaments at wavelengths of 532, 537 and 280 nm, and examples are shown in figs 8–10. The phase contrast microscope shows the lesion as a phase dense spot in the center of the phase lucent band. Polarization microscopy shows that laser irradiation results in the loss of birefringence from the target area (fig. 10). Electron microscopy of a lesion induced in a band of 100 Å filaments irradiated at 532 nm shows an electron dense core surrounded by a zone of disorganized filaments and filament fragments (see fig. 9). The dense spot measures 0.25 μm , and

Fig. 10. (a, b) Polarizing microscope picture of cell with band of 100 Å diameter filaments that was laser microirradiated at a wavelength of 280 nm. Two lesions are indicated by arrows. (a) Immediately fol-

lowing irradiation; (b) 3 h after irradiation. Band has changed position slightly, but lesions have not changed position relative to one another. $\times 1200$.

the total diameter of the dense spot plus the disorganized region which surrounds it is approx. 1–1.25 μm .

In order to determine whether or not movement takes place within the band of filaments, several lesions were made within the bands of individual cells and were observed via phase contrast or polarizing

microscopy for several hours following irradiation. Fig. 10 shows the result of one such experiment. Over the 3 h period during which the cell was followed, the shape of the band changed slightly, but the position of the lesions relative to one another remained constant. The results were the same in six similar experiments. Whether the multiple lesions were produced in an array parallel to or perpendicular to the long axis of the phase lucent band, neither the relative positions of the lesions nor the distances between them appeared to change over a period of 3–3.5 h. No movement resembling a contraction or retraction was observed immediately following irradiation of the band of intermediate filaments.

Fig. 9. Phase contrast micrographs of cell containing band of intermediate diameter filaments (a) prior to; (b) immediately following laser microirradiation at a wavelength of 532 nm. Arrow indicates lesion. $\times 2240$; (c, d) Low and high power electron micrographs of same cell as (a, b) showing laser-induced lesion. Note electron-dense spot at lesion center 0.25 μm in diameter, and the total diameter of the dense spot plus the disorganized region is 1–1.25 μm . (c) $\times 9500$; (d) $\times 25000$.

Fig. 11. Abscissa: wavelength (nm); ordinate: absorbance.

Plot of absorbance of soluble protein extracts in the range of 370–570 nm. Note discontinuity of abscissa between 450–510 nm. Absorbance of the three extracts plotted approaches 0 in this range. ●—●, Absorbance by commercially supplied horse heart myoglobin at 0.1 mg/ml in 0.05 M Tris-HCl, pH 7.5; to be read against left ordinate scale; ○—○, absorbance by soluble extract of whole neonatal rat heart; ×—×, absorbance by soluble extract of tertiary neonatal rat heart culture; both to be read against right ordinate scale.

Laser energy absorption biochemistry

Our ability to produce laser lesions in stress fibers in non-muscle cells using a wavelength of 537 nm led us to investigate the possibility that our cells contained the soluble pigment, myoglobin. Myoglobin has been reported to have two absorption peaks in the visible range, one at 413–415 nm and a smaller and somewhat broader peak at 540 nm [2]. The spectrophotometric results are shown in fig. 11. The two absorbance peaks of the myoglobin standard and the soluble extract of whole heart were evident while the extracts derived from tertiary cultures of neonatal rat heart did not show absorbance in these regions. Polyacrylamide gel electrophoresis was performed on the

same extracts used above. Myoglobin was evident in the whole heart extract and could be identified by virtue of its migration rate similar to that of the commercially supplied myoglobin standard. The fibroblast extract did not appear to contain detectable myoglobin, and thus it appeared unlikely that absorption by myoglobin accounted for laser energy absorption by the stress fibers in these cells.

DISCUSSION

We previously used the Nd-YAG laser irradiation system to investigate myofibrils and contractility in cultured cardiac muscle cells [1]. The presence of myoglobin made this system ideal for these studies due to absorbance by this soluble pigment in the green range of the visible spectrum, $\lambda = 537$ nm. The intracellular distribution of the pigment in the region of the myofibrils [11] allowed us to restrict damage to narrow portions of the myofibril with great precision. Most previous laser studies [12–14] of cardiac muscle cells had made use of the argon laser or ruby laser and were conducted primarily at wavelengths of 488 and 514 nm. In these cases, the targets were mitochondria and absorption of the laser energy was probably based on the well known absorption maxima of the various cytochromes contained in these organelles. In short, most laser irradiation studies of cultured cardiac cells have been restricted, at least in theory, by the necessity to match the laser wavelength used to an absorption peak. Vital dyes and photosensitizing agents, such as acridine orange [15–18], have been used as artificial chromophores in nuclear irradiations, but investigations of most cytoplasmic structures have been hampered by the small number

of sufficiently specific vital dyes. The present study indicates that this restriction does not always apply. Despite the lack of any known naturally occurring extramitochondrial chromophore in the cytoplasm of non-muscle cells with absorption in the 532 or 537 nm region, target structures were lesioned with a precision unavailable by conventional mechanical means. Damage produced at these visible wavelengths was identical in every respect to that produced by UV irradiation (280 nm) where absorption by biological molecules is well known. No direct evidence is presently available on the mechanism of laser damage at "non-absorbed" wavelengths though certain unusual physical phenomena have been postulated [19] to account for it. Relatively high photon densities, such as those produced by the Nd-YAG laser, appear to be required.

Thus, it appears that laser irradiation at visible wavelengths is relatively "non-specific" in biological systems in the sense that absorption may not be restricted to a particular class of molecules. On the other hand, the geometry of the focused beam and the Gaussian distribution of its energy provides sufficient precision for the type of experiments reported here. This conclusion is based on two lines of evidence. (1) TEM of laser irradiated stress fibers and bands of intermediate diameter filaments shows that obvious ultrastructural damage is limited to the target fiber or bundle. The diameter of the damaged zone can be controlled by neutral density filters and the properties of the objective lenses employed. If we assume that an average irradiated cell is roughly cylindrical, only 20 μm in diameter and 1 μm in thickness, and laser damage using appropriate neutral density filters and a 100 $\times$ objective is roughly spherical and approx. 0.5 μm in diameter, the cell's vol-

ume which appears in TEM to be affected would amount to 1/320 000th of its total volume. The significance of this damage to cellular economy would vary with the structures involved, but it might be small, especially if damage to the cell membrane can be prevented. (2) Preliminary experiments have been performed in which laser irradiation of stress fibers was followed by SEM. The scanning results clearly indicate that visible damage to the cell surface can be avoided and that laser damage probably involves heat transfer equal in all directions from the focused laser spot [20]. These data will be discussed at length in succeeding publications. Taken with the results of the present studies, the unique power and specificity of the laser can be applied to answer a variety of questions concerning subcellular cell functions.

The importance of microfilaments in non-muscle cell structure and motility has received considerable attention over the last several years (for reviews, see [21–24]). Fibers composed of 60 Å filaments have been identified in the cytoplasm of numerous cell types [25, 26, 27], and actin and myosin have been shown to be their major constituents [28, 29, 30, 33]. Glycerinated cell models [25] and isolated fibers contract in the presence of ATP; thus, the evidence that these fibers are responsible for cell movements is strong but circumstantial. The question arises as to whether the presence of contractile fibers always denotes contractility and cell motility. Non-muscle cell motility in heart subcultures appears to decrease with subculturing and time in culture (Strahs, unpublished observations); yet the number and thickness of the "stress fibers" clearly increase with these parameters.

The results of the stress fiber irradiations presented here implies that stress fibers are

of two types: "retracting" and non-retracting. It is not clear at the present time whether the retraction observed in some stress fibers is similar to a muscle contraction in which filaments slide past one another or whether this movement merely indicates that some fibers are under tension. The ability of the cut ends of some stress fibers to move following laser irradiation despite the presence of EGTA in the extracellular medium clearly distinguishes this phenomenon from laser-induced contractility changes found in cultured heart muscle cells [1]. If the retraction involves filaments sliding past one another away from the lesion, one might expect to see a thickening of the severed fiber ends. These measurements are difficult to make, and thus far, the results are equivocal. Recently, Lazarides [31] has shown that there are two classes of cytoplasmic actin filaments in cultured cells, one which binds tropomyosin and one which does not. Lazarides suggested that the presence of tropomyosin implies rigidity. Laser irradiation studies coupled with immunofluorescence might prove an interesting test of this hypothesis. These studies are presently in progress.

Microtubules do not appear necessary for the stability, the alignment or the repair of stress fibers; yet their structural apposition in the present studies is striking. Recent evidence by Fujiwara & Pollard [32] stresses the independence of the distribution of microtubules and myosin in well spread cultured cells and indicates that this apposition might be fortuitous. The colcemid resistance of the microtubules paralleling the fibers implies that these subunits are turning over at a slower rate than those of other cytoplasmic microtubules. It would be interesting to know whether the microtubule and myosin distribution is still in-

dependent following brief colcemid treatment.

The ability of bands of intermediate filaments induced by colcemid to move extensively has been documented [10]. Until now, however, little evidence was available to indicate whether the movement reflected intracellular cytoplasmic motility in which the bundles moved passively or whether the intermediate filaments could move with respect to one another. The results of the laser microbeam studies presented here seem to favor the former idea. The small areas of laser-induced filament disorganization and consequent loss of birefringence neither change shape nor change relative position during the first few hours of band movement. Longer term studies are presently underway in an attempt to follow specific regions of the bands to determine whether they are connected to other cellular structures, such as the nuclear membrane or the cell surface. We hope that these types of experiments will yield information concerning function of 100 Å filaments and subcellular movement.

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