Visualization of subcellular changes induced by CO_2-laser surgery in vitro

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Summary

Tissue damage by CO_2-laser surgery had been investigated extensively at a histological level in vivo and ex vivo. Here, the subcellular changes induced by CO_2-laser surgery have been documented in a tissue-like environment in vitro.
From the center of collagen gels populated with human fibroblasts a cylinder of 1 mm diameter was removed by CO_2-laser and compared to similar wounds inflicted by a stencil. Polymerized actin (F-actin) constituting the microfilaments of the cytoskeleton was labeled by fluorescent TRITC-phalloidin and visualized three-dimensionally by confocal laser scanning microscopy (CLSM). The integrity of the actin cytoskeleton served as an indicator for cell damage. Upon mechanical wounding, cells at the wound edge had contracted and rounded up. They lacked distinct actin fibers. Mechanical injury, known to initiate actin desintegration concomitant with a rounding up of the cells and shedding of growth factors, was used for comparison. In contrast, cells at the edge of CO_2-laser wounds, having protruded into the wound space had their actin cytoskeleton disintegrated or distorted, but without changing cellular shape. Cells farther off displayed an intact, though locally distorted, actin cytoskeleton. The zone containing visibly damaged cells extended about 100 µm from the wound edge perpendicularly into the intact tissue.
The lack of changes in cellular shape and the persistence of only locally distorted actin fibers indicate rapid denaturation of cellular structures, most probably also of intracellular growth factors and cytokines. Deactivation or lack of such internal factors by CO_2-laser may contribute to the reported delay of early tissue repair and also prevent hyperactive repair processes potentially leading to fibrosis and scarring.

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Introduction

The CO_2-laser is replacing conventional techniques such as scalpel surgery or diathermy especially in oral surgery (Frame 1985a, b; Rhys Evans et al. 1986, Nemeth 1993, Pick & Colvard 1993, Clayman & Kuo 1997). The CO_2-laser operates in the infrared range at a wavelength of 10.6 µm. Thus, its energy is specifically absorbed by water molecules, which causes the water-rich mucous to be preferentially vaporized. The most obvious advantages of CO_2-laser surgery are precise destruction of tissue with minimal bleeding, the initial absence of major pain and tissue swelling, and reduced scarring (Frazell & Lucas 1962, Strong et al. 1973, Ben-Bassat et al. 1978, Strong et al. 1979, Carruth 1982, Dhillon et al. 1982, Vaughan 1982). These beneficial effects seem to be balanced by slower wound closure in comparison to that initiated by mechanical wounding (Pick & Colvard 1993). The reduced inflammatory reactions go along with delayed collagen synthesis and epithelization, and reduced tensile strength in the early phase of repair. In the later stages of tissue repair these early differences are generally compensated for, eventually leading to a comparable quality of tissue repair (Hendrick & Meyers 1995).
Experimental laser surgery had been performed on various types of excised tissue and on animals, and also in comparison to conventional scalpel surgery, and in relation to clinical data

(Fisher et al. 1983, Zweig et al. 1990, Pogrel et al. 1990ab, Pick & Colvard 1993, Pinheiro et al. 1993, 1995, Keller et al. 1995, Hendrick & Meyers 1995, Evrad et al. 1996). The induced effects had been documented by histology in relation to the applied energy and the induced thermal damage. Only recently, laser-induced biochemical changes have been investigated in conventional two-dimensional cell cultures (Nowak et al. 2000), and compared to the effects of mechanical tissue injury simulated in vitro by scratching such cultures (Todaro et al. 1965, Schreier et al. 1993), In the present study, cells were cultivated as dermal equivalents as (Bell et al. 1979) to provide a tissue-like environment. Baschong et al. (1997) had used such a culturing system for emulating the natural three-dimensional aspect of connective tissue when investigating the fate of cellular form and function upon mechanical injury by means of confocal laser scanning microscopy (CLSM). Here, this same system has been used for three-dimensional visualization of immediate cellular changes induced by CO_2-laser surgery.

Material and Methods

Human skin fibroblasts were grown in fibroblast-populated collagen gels as described in detail by Baschong et al. (1997, 1999). In short, lip-derived KD-fibroblasts (ATCC CRL 1295, American Type Culture Collection, Rockville, MD 20852) in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO 041–01885; Life Technologies, Gaithersburg, MD 20877) (pH 7.2–7.4) supplemented with 20% fetal calf serum (FCS) and 100 units/ml Penicillin 100 µg/ml Streptomycin (Pen/Strep, 100× stock solution; GIBCO). Cells were grown at 37 °C in a humid atmosphere of 95% air/5% CO_2. Cell detachment was done by trypsin/EDTA. The collagen gels were prepared as attached low-contracting dermal equivalents (ALDE) by mixing at ambient temperature 1.5 ml of 10× DMEM (GIBCO), 2.8 ml of 0.1 N NaOH, 150 µl of Pen/Strep stock solution, 150 µl of 0.2 M glutamate (GIBCO), 150 µl of 0.1 M sodium pyruvate (GIBCO), 120 µl of 7.5% $NaHCO_3$, 375 µl of 1 M HEPES and 1.5 ml of FCS, and combining it with 8.25 ml of Vitrogen 100 solution (Type I collagen; Collagen Corporation, Palo Alto, CA) and 2.105 cells/ml (suspended in 1/50 of the final volume of DMEM containing 10% FCS and 1/100 Pen/Strep stock solution). 0.5 ml portions of this mixture (15 ml) were subsequently added to the wells of 24-well culture plates. Culture plates were left for gelation in a warm room for 1 hour at 37 °C, and then transferred to a 37 °C incubator (95% air/5% CO_2).

Wounds were inflicted two days post seeding by excising a cylindrical "biopsy" of about 1 mm in diameter from the center of the ALDE, either by using a sterilized Pasteur glass pipette as a stencil, or by using a cw CO_2-laser (GMP, Ceram Optec, Bonn, Germany). The settings of the CO_2-laser were optimized for energy and duration of the pulse for each series of ALDE. Criterion were i) complete removal of a tissue cylinder with a diameter of 1 mm and perpendicular to the surface and ii) no damage to the underlying plastic bottom. Optimal settings ranged between 0.1 sec at 5 W and 0.1 sec at 20 W, or 0.2 sec at 10 W respectively, with the focus set accordingly. Matrices were detached and processed for labeling immediately (within less than 5 minutes) after wounding. All experiments were carried out at least in triplicate.

Permeabilization, fixation, and fluorescence labeling of ALDE was performed at room temperature as described in detail by Baschong et al. (1997, 1999). Wounded ALDE or controls were detached from the side wall of the cell culture well and transferred into wells containing 0.5–1 ml/well of DMEM supplemented with 10% FCS. This buffer was then replaced by modified Hanks' buffer (MHB: Ca-free, containing 2 mM EGTA and 5 mM MES [2-morpholino-ethanesulfonic acid], pH 6.2–6.4); and then quickly substituted by permeabilization buffer, i.e. with MHB additionally containing 0.125% glutaraldehyde and 2% octyl-POE (n-octylpolyoxyethylene). The time from detachment to permeabilization was kept under 3 minutes. After 15 minutes of permeabilization and prefixation, cells were fixed for 20 minutes with MHB containing 1% glutaraldehyde which was then replaced 3–4 times by MHB before aldehyde groups were reduced by twice treating for 10 minutes with 0.5 mg/ml of NaHB4 in MHB on ice. ALDE were stored at 4 °C in MHB containing 0.005 % sodium azide.

Permeabilized and fixed ALDE were incubated in MHB for 3 hours at room temperature with TRITC-phalloidin (Sigma, St. Louis MO, USA) and followed by washing three times with MHB.

The labeled specimens were mounted bottom-up in Mowiol-4-88 (Hoechst, Frankfurt BRD), i.e. the gels were inverted prior to mounting. Mounted slides were stored at 4 °C in the dark until viewed.

Conventional phase contrast microscopy was performed with a Zeiss IM3 invert microscope and conventional fluorescence microscopy (CFM) with a Zeus Axiophot fluorescence microscope.

Confocal laser scanning microscopy (CLSM) was done on a Zeiss Axiovert-135 microscope equipped with a Noran-Odyssey (Noran, Middleton, WI, 53562) confocal device and using a 63× Zeiss Plan-Apochromat objective with a numerical aperture of 1.4, or with a Leica TCS 4D CLSM with a Leitz 63x objective with an aperture of 1.3. The resolving power for fluorescence imaging was thus approximately 0.35 µm in x-, y- and z-direction. Optical sections were processed and 3-D images computed by Image-1 software (Universal Imaging Corp. West Chester, PA 19380) or by Imaris software (Bitplane, Zurich, Switzerland). The wavelength of the intrinsic fluorescence of the collagen fibers forming the matrix did not interfere, neither with conventional fluorescence microscopy nor with confocal laser scanning microscopy.

Results

Human fibroblasts cultivated for two days in three-dimensional collagen gels and under attached low-contracting conditions were used as tissue substitutes. Figure 1 displays such dermal equivalents in the culturing plate (Fig. 1A) and the distribution of the fibroblasts within such a collagen gel. The latter was revealed by conventional fluorescence microscopy (CFM) after labeling with TRITC-phalloidin, a fluorescently labeled drug which binds specifically to polymerized actin. Fibroblasts had arranged as stellate to very elongated structures and parallel to the bottom of the culturing well, with their actin positive processes extending up to several hundred micrometers.

The morphology of these cells upon mechanical wounding and without fixation is illustrated in Fig. 2. The phase-contrast image in Fig. 2A displays cells in a collagen gel two days after seeding and one hour after mechanical wounding. At the wound edge cells had mostly rounded up while farther off the wound they displayed the stretched-stellate to bipolar morphology, as in the non-wounded control (Fig 2C). For the visualization of subcellular changes, collagen gels were again cultivated for two days and then wounded and fixed within five minutes of injury. These cells (Fig. 2B) and non-wounded controls (Fig 2D) were labeled

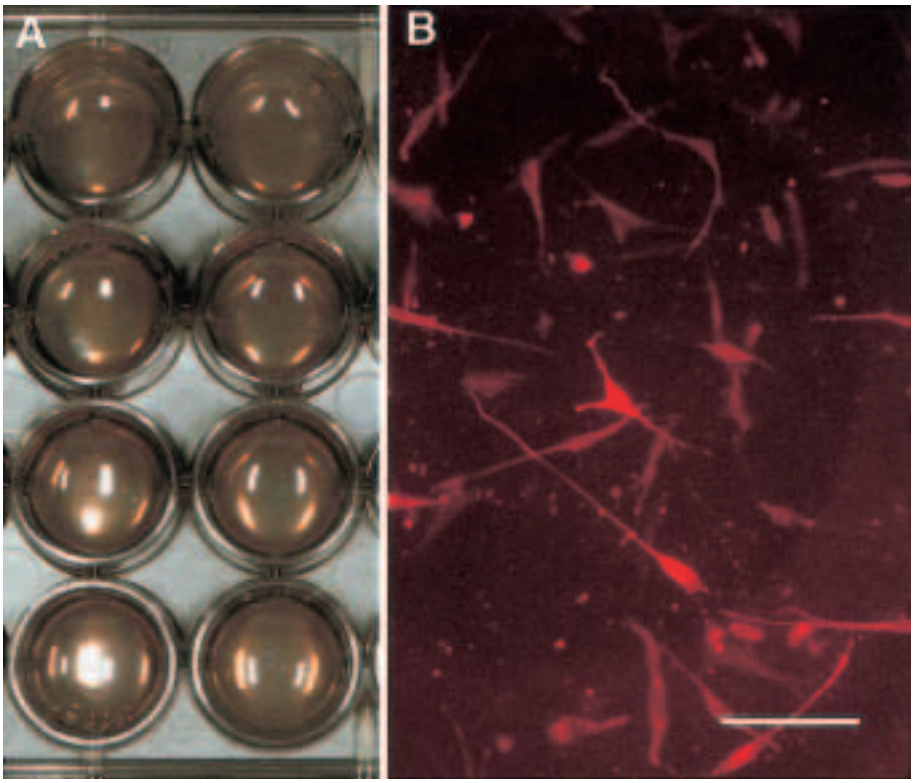

Fig. 1 Three-dimensional cell cultures in collagen gels:
A: Aspic-like collagen gels containing human fibroblasts in culturing wells (Ø 15 mm).
B: Distribution of fibroblasts within collagen gel revealed by TRITC-phalloidin labeling and conventional fluorescence microscopy (CFM). Scale bar 200 µm.

by TRITC-phalloidin, scanned by CLSM via virtual sectioning along the z-axis, and documented as composite images projected in the xy-plane: The actin cytoskeleton in these mechanically wounded collagen gels had disappeared, concomitant with the appearance of a high concentration of phalloidin-positive, but indiscernible structures. These cells had contracted or rounded up (Fig. 2C). In contrast, the actin in non-wounded cells formed bundles of actin fibers stretching into the cytoplasm and extending into the processes (Fig. 2D).
The morphological and subcellular changes induced by CO_2-laser wounding are documented in Fig. 3. The CFM overview in Fig. 3A displays fibroblasts grown for two days in collagen gels as above and injured by a CO_2-laser: Though charring of cells was not evident, cells in the wound zone had curled, i.e. were obviously damaged. Farther off the wound edge, cells were elongated without obvious curling and shaped like cells in non-wounded controls (Fig. 1C).
Analysis by CLSM revealed in the wounded zone three main types of structurally damaged cells: i) Cells, which had obviously been cut off showing a locally disintegrated actin cytoskeleton with many actin-positive remnants at the very wound edge and with the rest of the cell body exhibiting an essentially intact but locally distorted actin-cytoskeleton. (Fig 3B). ii) Cells demonstrating clear actin fibers together with a background of non-structured F-actin (Fig. 3C), with a shape comparable to that in non-wounded control gels (Fig. 1D). Such actin fibers showed local distortion or were undulated iii) Cells exhibiting distinct actin fluorescence along the fibers and very little background, giving the impression of an emptied cell body with a remaining actin scaffold (Fig 3D). Contracted cells or cells without actin filaments as encountered upon mechanical wounding were never observed. Damaged cells distributed in a zone that was estimated from micrographs to extend about 100 µm or more perpendicular from the wound edge about 100 into the intact tissue. In cells farther off, distinct signs of structural damage were absent, though thermal coagulation and denaturation of intracellular structures under preservation of a native-like morphology could not be excluded.

Discussion

The present study visualized the effects of CO_2-laser injury to single cells and their internal structures such as the actin microfilaments of the cytoskeleton in vitro, in a tissue-like environment and immediately upon injury in a system introduced for the study of mechanical stress-induced spatial cytoskeletal changes (Baschong et al. 1997, 1999). Within these three-dimensional cultures, unharmed cells assumed an elongated-stellate to stretched-bipolar shape, comparable to that in vivo, where sessile fibroblasts extend within the extracellular matrix in a three dimensional pattern and without extensive cell contact (Bell et al. 1979, Baschong et al., 1997). Extensive actin stress fibers such as in a cell monolayer culture were absent (Baschong et al. 1997).
Comparison of CO_2-laser wounds to similar-sized stencil wounds revealed the lack of mechanical stimulation upon laser injury. While mechanical wounding initiates cells at the wound edge to round up concomitant with a temporarily lack of an organized actin cytoskeleton (Unemori & Werb, 1986, Bouissou et al. 1988, Baschong et al. 1997), cells in laser-injured collagen gels maintained their overall stellate to extended shape, without signs of a response by contraction. CO_2-laser damage disintegrated actin fibers only at the very wound edge. Further off, actin filaments were distinctly visible, though sometimes locally distorted, indicating the absence of contraction. Contraction would manifest in the immediate de-polymerization of the actin cytoskeleton (Baschong et al. 1997).
Locally damaged actin fibers have been observed in a zone peripheral to the wound and extending about 100 µm from the wound edge. A similar-sized zone was described by Pogrel et

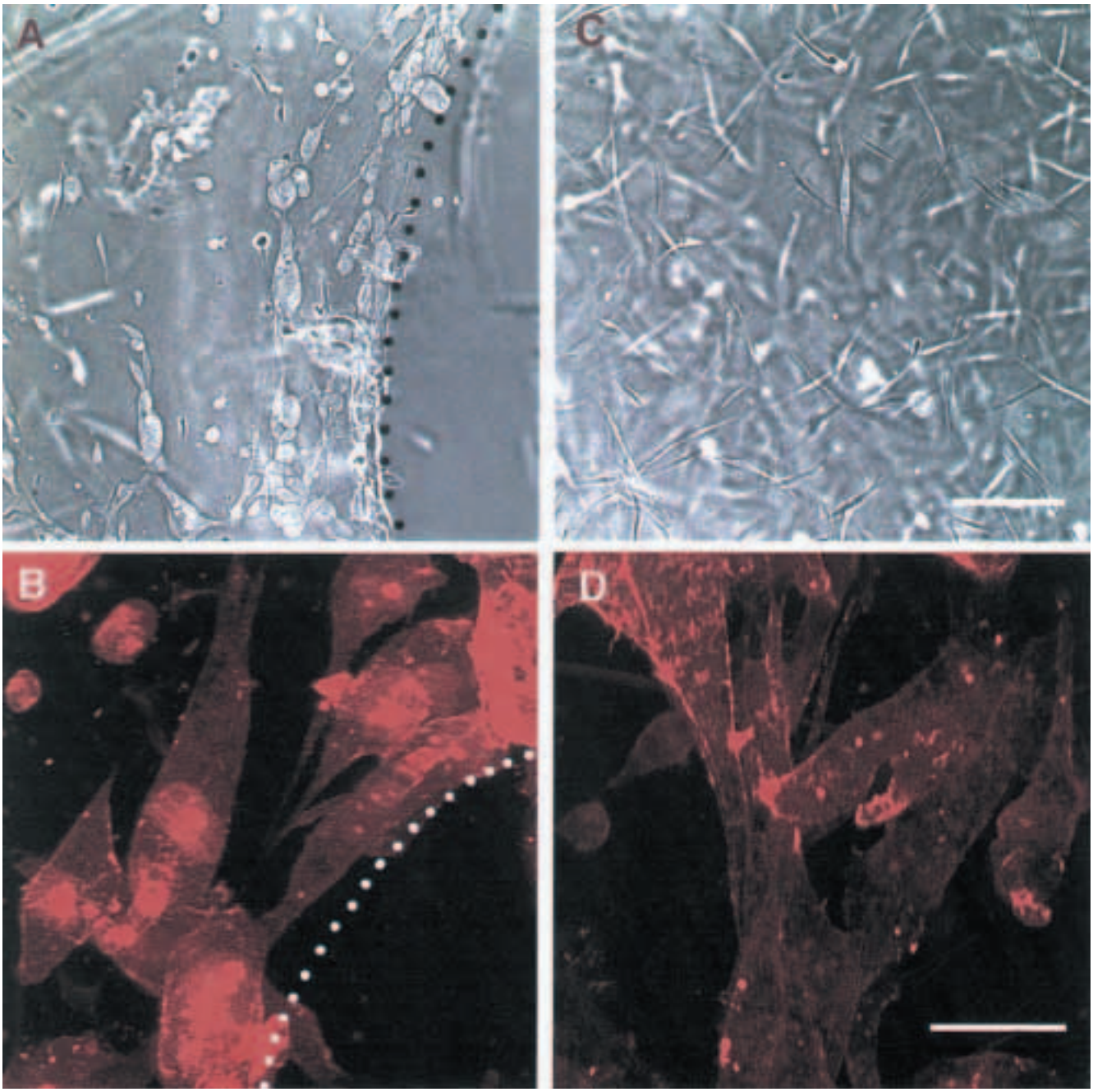

Fig. 2 Morphology and actin cytoskeleton of three-dimensionally grown fibroblasts. Fibroblast populated collagen gels were cultured for two days prior to wounding by mechanical excision of a 1 mm tissue cylinder. The dotted line indicates the wound edge.
A, C: Phase contrast images of collagen gels in culture: (A) 1 h upon mechanical wounding. C: non-wounded control. Scale bar 200 µm.
B, D: Confocal laser scanning micrographs (CLSM) of fibroblasts in collagen gels fixed by glutaraldehyde and within 5 min upon wounding. Cells were labeled fluorescently for filamentous actin by TRITC-phalloidin. CLSM composite images, i.e. overlays of n optical sections of 0.5 µm thickness (step size) registered along the z-axis.
B: immediately (< 5 min) upon wounding (n = 73, step size 0.5 µm).
D: non-wounded control (n = 110, step size 0.5 µm). Scale bar 20 µm.

al. (1990b) based on histology. Accordingly, KARDOS et al. (1989) described the zone of obvious structural alteration to extend between 50–100 µm, depending on the type of tissue investigated. BRYANT et al. (1998) estimated in canine oral mucosa and based on collagen denaturation, the thermal damage induced by CO_2-laser surgery to extend between 200–400 µm laterally and vertically from the site of incision.

Mechanical damage by crushing, tearing or a surgical cut mainly relates to the destruction of the extracellular matrix surrounding the cells, but changing cell shape and function in a reversible

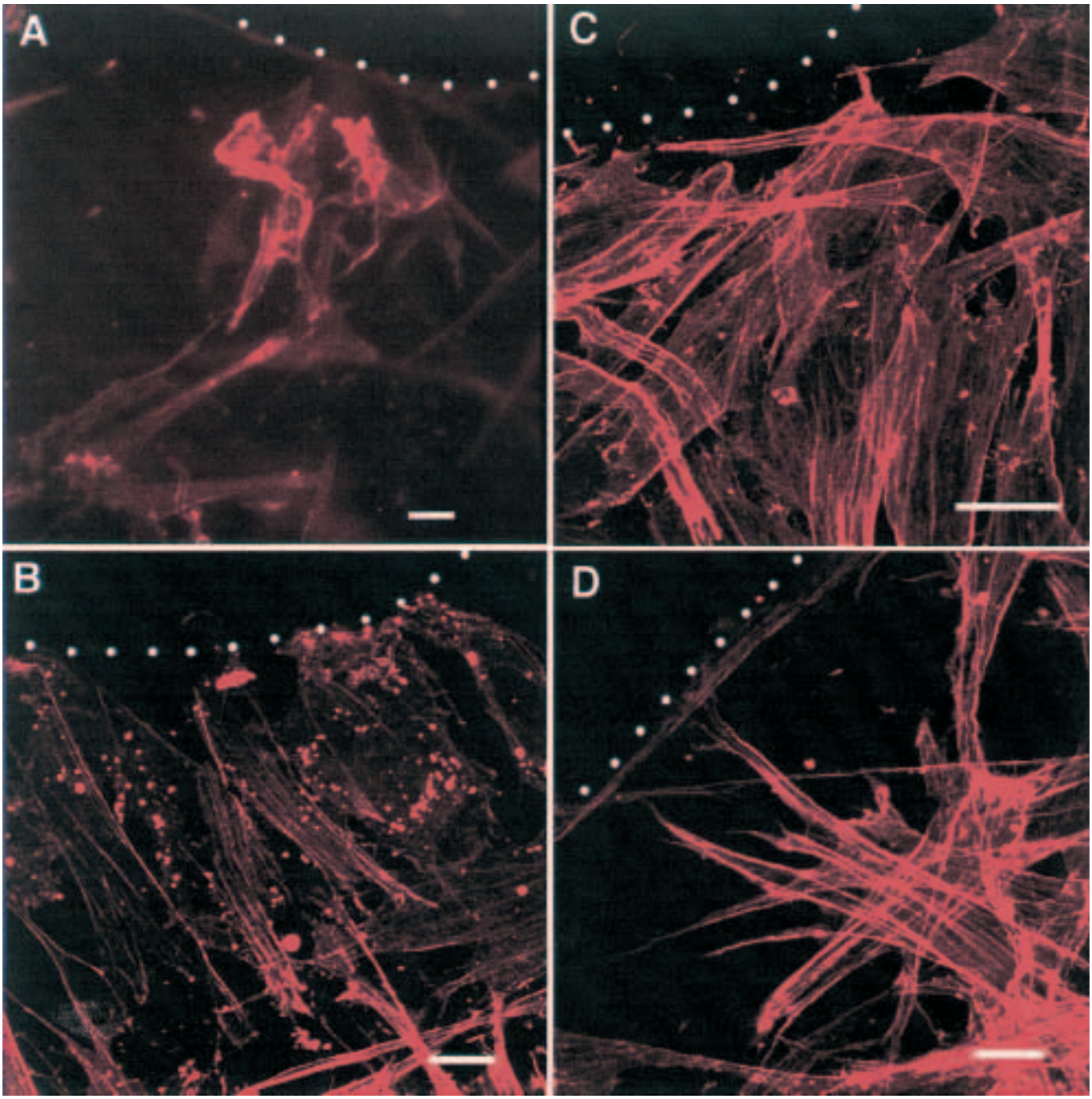

Fig. 3 Cell morphology and architecture of the actin-cytoskeleton of three-dimensionally grown fibroblasts. Fibroblast populated collagen gels were cultured for two days prior to excising a 1 mm tissue cylinder by CO_2-laser. Collagen gels were fixed with glutaraldehyde and then labeled for filamentous actin by TRITC-phalloidin (cf. Figures 2B, 2D). The dotted line indicates the wound edge.
A: Conventional fluorescence micrograph showing apparently distorted cells at the wound edge and elongated-stellate cells more distant from the wound. The arrangement of actin fibers is not resolved. Scale bar 200 µm.
B, D: CLSM composite images registered along the z-axis. CLSM-images reveal typical forms of laser damaged cells.
B: Cells with extensions (most probably in contact with the laser path) apparently disintegrated leaving remnants of actin-positive granules. Cells demonstrating rather intact but slightly distorted actin fibers with absence of an actin-positive background. N = 60 optical sections, 0.4 µm thick (step size). Scale bar 20 µm.
C, D: Cells arranging at the wound edge demonstrating a rather intact actin cytoskeleton extending into the cell processes and an actin-positive background comparable to non-wounded cells (cf. Fig 2D) and cells with distinct locally distorted or undulated actin fibers.
C: n = 15, step size: 0.4 µm; D: n = 35, step size: 0.3 µm. Scale bar 20 µm.

manner (Baschong et al. 1997). Such mechanically challenged cells had been observed to release metallo-proteinases (Unemori & Werb 1986) together with peptide growth factors (Tsuboi et al. 1990) stimulating tissue repair. In crush-injured tissue increased levels of PDGF (platelet derived growth factor) (Antoniades et al. 1991) and bFGF (basic fibroblasts growth factor) (Jackson & Reidy 1993) had been reported. bFGF had been detected already 3–6 hours after wounding (Anderson et al. 1995). These signals were demonstrated to diffuse to and activate also neighboring intact cells (Sammas et al. 1997). In contrast, with CO_2-laser surgery where the wound zone obviously "freezes" immediately upon injury, i.e. is thermo-coagulated, such mechanical stimulation is obviously absent, or reduced. The presence of metallo-proteinases and growth factor-cascades governing tissue repair may thus be limited and derive only from heat-activated cells (Nowak et al. 2000) in the zone of reversible heat damage, or as a secondary effect, from mechanical stimuli induced by tissue retraction. In conjunction with minimal bleeding, the lack of such dynamically released factors may contribute to delayed debridement, inflammation and epithelization, which may be beneficial in situations potentially leading to fibrosis and scarring, and even out during wound granulation and tissue remodeling.

In conclusion, the effect of CO_2-laser on single cells and subcelluar structures could be visualized in vitro, in a three-dimensional tissue-like environment and related to that in similar wounds obtained by scalpel surgery. The integrity of the actin cytoskeleton proved a useful marker for monitoring cell destruction and the dynamics of the cellular response to wounding.

Zusammenfassung

Durch CO_2-Laser-induzierte Gewebsschäden wurden eingehend in vivo und ex vivo, an explantiertem Gewebe, histologisch untersucht. In der vorliegenden Studie wurde der Effekt des CO_2-Lasers auf subzellulärer Ebene dreidimensional in einem gewebsähnlichen Verband kultivierter Zellen dokumentiert.

Aus der Mitte eines menschlichen Fibroblasten enthaltenden Kollagen-Gels wurde mittels eines CO_2-Lasers und zum Vergleich mechanisch mit einer Stanze ein Zylinder mit einem Durchmesser von 1 mm entfernt. Polymerisiertes Aktin (F-Aktin), wie es besonders in den Mikrofilamenten des Zellskelettes vorkommt, wurde mit dem fluoreszierenden Phalloidinderivat FITC-Phalloidin markiert. Die dreidimensionale Organisation der Aktinfilamente wurde mittels konfokaler Laser-Raster-Mikroskopie (CLSM) dargestellt. Der Zustand des Aktin-Zellskelettes diente als Indikator für Zellschäden: Im Gegensatz zu mechanischer Schädigung, auf welche Zellen sofort durch Kontraktion und Verlust einer sichtbaren Organisation des Aktinskelettes reagieren, veränderten Zellen nach Laserverletzung ihre äussere Form nicht. Zellen am Wundrand, die ursprünglich bis in die Wundzone vorgestossen waren, waren bis zum Wundrand hin verdampft. Zellen in der Wundzone zeigten keine Anzeichen von Kontraktion und waren in ihrer Form vergleichbar mit Zellen in intakten Kontrollkulturen. Ihr Aktin-Zellskelett war zwar lokal zerstört oder geschädigt, aber immer als solches erkennbar. Die Zone mit sichtbar geschädigten Zellen erstreckte sich über einen Radius von etwa 100 µm um die Wunde.

Die lokale Desintegration und Torsion der Aktin-Fibern weisen auf eine rasche Denaturierung der internen Strukturen hin, darin inbegriffen höchstwahrscheinlich auch intrazelluläre Wachstumsfaktoren und Zytokine. Gerade das Fehlen oder die Desaktivierung solcher Faktoren könnte einerseits zu einer Verzögerung der frühen Phase der Wundheilung beitragen, andererseits aber auch ein Überschiessen von Reparaturprozessen, die zu Fibrosis und extremer Narbenbildung führen können, verhindern. Beide Prozesse, ein späteres Einsetzen der Wundheilung und eine bessere Prognose im Bezug auf Narbenbildung wurden in der Klinik nach Verwendung des CO_2-Lasers beobachtet.

Résumé

Les dommages tissulaires induits par la chirurgie à laser CO_2 ont été étudiés, intensivement, au niveau histologique tant in vitro qu'ex vivo. L'étude présentée ici documente les effets de cette technique chirurgicale au niveau subcellulaire dans un modèle tissulaire in vitro. Ce modèle consistait en des cellules cultivées en trois dimensions dans un gel de collagène. Un cylindre de 1 mm de diamètre était prélevé, par la technique du laser CO_2, au centre d'un gel de collagène colonisé par des fibroblastes humains et était comparé à un prélèvement mécanique similaire effectué à l'aide d'un pochoir. L'actine polymérisée (F-actin), constituant des microfilaments du cytosquelette, était marquée à l'aide du dérivé fluorescent TRITC-phalloïdin puis visualisée en trois dimensions par microscopie CLSM. L'état du cytosquelette, après prélèvement, était un indicateur des dommages infligés au niveau subcellulaire. Après prélèvement mécanique, les cellules en bordure du cylindre, c'est-à-dire dans la zone de blessure, étaient contractées et arrondies. L'actine du cytosquelette n'était plus visible. Ces observations étaient en accord avec les effets des blessures mécaniques précèdemment décrits. Effectivement, celles-ci entrainaient une désintégration du cytosquelette, un arrondissement des cellules et une augmentation du relargage de certains facteurs de croissance.

Au contraire, bien que présentant un cytosquelette détruit ou localement désorganisé, les cellules de la zone de blessure, induite par le prélèvement au laser CO_2, n'avaient subi aucunes modifications de forme. Les cellules d'alentours présentaient un cytosquelette soit localement plié, soit intact. La zone contenant des cellules visiblement endommagées s'étendait depuis la bordure du cylindre jusqu'à environ 100 µm perpendiculairement à l'intérieur du gel.

La désintégration locale du cytosquelette sans modifications de la forme des cellules semblait indiquer une dénaturation très rapide de la structure cellulaire par le laser CO_2. Potentiellement, ceci pourrait se traduire par une inhibition du relargage des facteurs de croissance et des cytokines expliquant, en partie, le retard de guérison tissulaire fréquemment observé en clinique suite à l'utilisation du laser CO_2. De plus, cette absence de relargage pourrait prévenir la cicatrisation non contrôlée conduisant à la fibrose.

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