PULSED HIGHPOWER-LASER RADIATION INDUCES CELL PROLIFERATION AND INCREASED SYNTHESIS OF THE EXTRACELLULAR MATRIX COMPONENTS IN CULTURED HUMAN CHONDROCYTES

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Introduction

The articular cartilage (AC) is a differentiated aneural, alymphatic and avascular tissue, with scarce cellularity and a well developed extracellular matrix (Dijkgraaf et al., 1993; Muir et al., 1995), which posses a limited capacity for re-growth and repair after injury (Buckwalter, 2002).

The therapeutic strategies for treatment of AC pathologies include drugs (Hochberg and Dougados, 2003), surgery (Gross, 2003), autologous transplantation of cultured chondrocytes (Brittberg et al., 2003; Wang et al., 2004), growth factors (Holland and Mikos, 2003) and gene therapy (Gelse et al., 2003). Furthermore, exposition to pulsed electromagnetic fields (Fioravanti et al., 2002; Fini et al., 2005) and laser radiation (Cho et al., 2000; Morrone et al., 2000; Lu et al., 2001) have been used with a variable success for the treatment of these pathologies.

A new pulsed high-power laser (PHPL) has been introduced recently in medicine, which is able to induce analgesia (manufacturer's notice) and improve the osteoarthritis induced in chicken (P. Mondardini, personal communication of unpublished results). However, the cellular and molecular mechanisms regulating these effects are unknown. The present study was undertaken to analyze the effects of two different doses (50 and 75 mW) of PHPL on cultured human chondrocytes.

Material and Methods

Tissues.- Samples of human AC (n = 2) were obtained from two adult healthy subjects, 1 male and 1 female, aged 26 and 41 years respectively, who had died in traffic accidents, during removal of organs for transplantation. The pieces were collected in cold physiologic-isotonic solution and processed for tissue digestion in order to isolate chondrocytes. The material was obtained in compliance with Spanish Law for brain death and organ transplantation (Law 30/79 and Royal Decree 426/80 and 411/96), and Helsinki II.

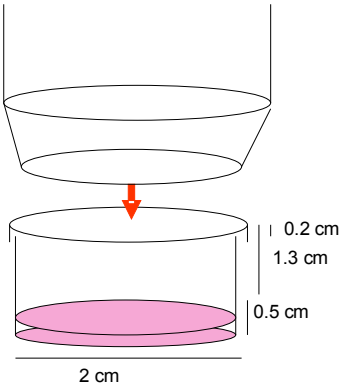
AC digestion and isolation of chondrocytes.- The samples were cleaned with Ham's F12 sterile (Gibco/BRL), and then exposed to enzymatic digestion with 2,5% Tripsin (Sigma, USA) for 20 minutes at 37º C. Subsequently, the pieces were submitted to mechanical disruption followed by a new enzymatic digestion with collagenase (2 mg/ml; Sigma) for 16-20 hours at 37º C in 5% CO_2. The resulting product was centrifuged (1400 r.p.m.) for 10 minutes, and the pellets re-suspended, and placed into the culture media. The viability of the cells was confirmed by means of the tripan bleu test (Sigma).

Cell culture.- The isolated chondrocytes were incubated for 30 days in a medium containing 10% FBS, 1% penicillin-streptomicin, 50 μg/ml ascorbic acid, and 1% L-glutamin, and were used as a control. From cultures, an average of 10.000 cells/ml were obtained, and cultured in parallel in order to valuate cell growth curves, or to obtain cells for secondary culture.

Representative samples of cultured chondrocytes, mean average of 10.000 cells/ml, were processed for secondary culture in the same medium. All the experiments were carried out in a culture chamber at 37 ºC, 5% CO_2, and 95% of humidity. The cells were controlled daily and the medium were renewed each 3 days.

Laser radiation.- Secondary cultures of chondrocytes were radiated with a FIBER LASER ICL60 PLUS HFPL 250, at the following total dose of irradiation: a) 50mW (250Watt, 100ns, 2kHz, time for 25 joules = 8,3 min); b) 75mW (250Watt, 100ns, 3kHz, time for 25 joules = 5,5 min). The interface between the laser source and the

liquid containing the chondrocytes was 2 mm of plastic-glass, 11 mm of aerial space. The radiation was identical and uniform in the entire surface.



Analysis of the cell growth kinetics.- Secondary cultures of chondrocytes (x10), radiated and controls, were sampled in duplicate at the beginning of the experimentation and each 48 h (x2) to determine the cell growth rate. The cultures were digested with Tripsin-EDTA (0,05-0.02%; Sigma) for 2 m at 37º C, them centrifuged at 1.400 r.p.m. for 10 m, and the supernatant and the pellet re-suspended. Cells were counted using an Enerbauer's camera.

Determination of the cell cycle: flow cytometry.- At the end of the experiments cell samples were obtained from secondary cultures radiated and controls, concentrated through centrifugation and incubated with iodide. Thereafter, the pellet was re-suspended in a medium containing trypsin and RNAase inhibitors. Finally, it was incubated in dark at room temperature with propidium iodide, and analyzed by FAC flow cytometry with an Argon laser at 488 nm.

Detection of apoptosis: caspase-3 activity assay.- Caspase-3 activity was measured using the BD Apoalert™ Caspase-3 Assay (BD Bioscience Clontech, La Jolla, CA) following manufacturer's instructions. Samples of the control and radiated secondary cultures of chondrocytes were obtained 4 days after irradiation,

and they were separated from the culture plate using trypsin and incubated for 10 minutes with lysis buffer. Cellular debris were discarded by centrifugation for 10 minutes at 4°C. Each sample was divided into three parts and one of then incubated with the caspase-3 inhibitor DEVD-CHO as negative control for 30 minutes. All the samples were subsequently incubated with 50 µl of 2X reaction buffer containing 10 mM DTT and 50 mM caspase substrate DEVD-AFC at 37°C for one hour. One of the two samples in which we did not inhibit caspase-3 activity was not incubated with caspase substrate. Samples were transferred to a 96 well plate and analyzed using a fluorometer with 400 nm excitation and 505 nm emission filters.

Gene expression analysis.- Samples of secondary cultures (x3) radiated and controls were frozen in liquid nitrogen. After removing the culture medium, cells were weighed and then processed using the Mikro-Dismembrator S (B. Braun Biotech International GmbH, Germany). RNA was extracted using RNA-Bee™ (TEL-TEST, Inc; Friendswood, USA) according to manufacturer's guidelines and subsequently precipitated with 2-propanol. RNA was further purified using RNeasy Micro Kit (Qiagen, The Netherlands) with on-column DNA-digestion. Total RNA was quantified accurately using Ribogreen reagent (R-11490, Molecular Probes Europe BV, The Netherlands) according to manufacturer's instructions and 500 ng total RNA of each sample was reverse transcribed into complementary DNA (cDNA) using RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). Primers and probe sets were designed using PrimerExpress 2.0 software (Applied Biosystems, USA) and were designed to bind to separate exons to avoid false positive results arising from amplification of contaminating genomic DNA. BLASTN search was used to ensure gene specificity of all oligo-nucleotide sequences. The primer and probe nucleotide sequences are listed the following table.

Primer and probe nucleotide sequences of the tested genes

Col2	Fw: GGCAATAGCAGGTTCACGTACA Rv: CGATAACAGTCTTGCCCCACTT	CCGGTATGTTTCGTGCAGCCATCCT
AGC1	Fw: TCGAGGACAGCGAGGCC ATGGAACACGATGCCTTTCACCACGA	ATGGAACACGATGCCTTTCACCACGA
MMP2	Fw: TCAAGTTCCCCGGCGAT Rv: TGTTCAGGTATTGCACTGCCA	CACAACTGCCAAATGGGCTTGAAGC
MMP13	Fw: AAGGAGCATGGCGACTTCT Rv: TGGCCCAGGAGGAAAAGC	CCCTCTGGCCTGCGGCTCA

Measurement type II collagen, proteoglycans, integrins and MMPs.- The measurement in the culture medium of type II collagen, protyeoglycans, integrins and MMPs was done by two-site ELISA as follows: 96-well plates were coated with a polyclonal antibody against each of the assessed antigens or with a rabbit preimmune serum. After incubation for 12h at room temperature, the wells were incubated for 2h with blocking buffer (2% bovine serum albumin in 0.05M bicarbonate buffer, pH 9.5). The wells were then washed repeatedly (0.05M Tris-HCl buffer, pH 7.4, containing 200mM NaOH, 5% gelatine and 0.1% Triton X-100) and incubated with the sera and the standards for 12h at room temperature. After rinsing, each well was incubated with 4mU anti each specific antigen-β-galactosidase (Boehringer-Mannheim, Germany) for 2h at 37ºC. Wells were washed again and incubated with 100μl phenol red solution (4mg/ml) for another 2 hrs at 37ºC. Finally, the optical density at 575 nm was measured using an ELISA reader and the values of the standards were corrected subtracting the value of the controls, considered as non-specific reaction.

Westernblot.- Western-blot analysis was performed in samples containing both chondrocytes and culture medium. The samples were homogenized mechanically and subsequently lysed on ice in 1% digitonine-containing lysis buffer (1% digitonine, 50mM Tris-HCl, 150mM NaCl, 1mM $MgCl_2$, 0.1 mM EDTA, 8mM iodoacetamide, and 1mM phenylmethylsulfonyl fluoride; pH7.6; Zapata et al. 1999). Then homogenates were centrifuged at 6000xg for 10 min at 4º C, and the supernatants collected and centrifuged for 10 min 4º C at 14000xg to eliminate the

smallest cell debris. The resulting pellets were re-diluted and separated in SDS-page (10%), and the proteins electrophoretically transferred to a nitrocellulose sheet. The sheet was then blocked with Tris-buffer saline (TBS, 1M, pH 7.4) containing 1% fat free milk and 1% Tween-20, and incubated for 1 h at room temperature with the primary antibodies. The transblot was rinsed with TBS and 1% tween-20 and incubated with peroxidase-labeled anti-rabbit IgG or anti-mouse IgG (Amersham) both diluted 1:1000 for 1 h, washed, and analyzed by the ECL Western blotting detection system (Amersham).

Immunohistochemical analysis.- The expression of type II collagen, proteoglicans, integrins, insulin-like growth factor receptor type I, and metalloproteases in the cultured cells was evaluated by immunohistochemistry. Cell were fixed in cold acetone, air dried, and rehydrated. After bocking non-specific binding (25% foetal calf serum) and endogenous peroxidase activity (3% H_2O_2), cells were incubated with the primary antibodies over-night in a humid chamber, at 37º C. Thereafter, the cells were washed and then incubated for 1 h at room temperature with peroxidase-labeled sheep anti-rabbit IgG or anti-mouse IgG (Amersham, Buckinghamshire, UK), both diluted 1:100. Finally, the cells were rinsed and the immunoreaction visualized using 3-3'-diaminobenzidine as a chromogen. For control purposes, cell samples were processed in the same way omitting the primary antibody in the incubation, or using a non-immune rabbit or mouse serum instead of the primary antibody. Under these conditions, no specific immunostaining was observed.

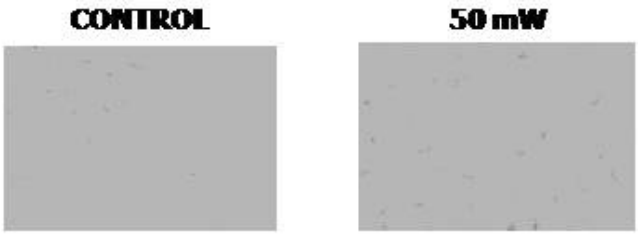
Morphological study.- In cultures at the ending of the experiments, 4 days after radiation, the morphology of the chondrocytes well as of the synthesized

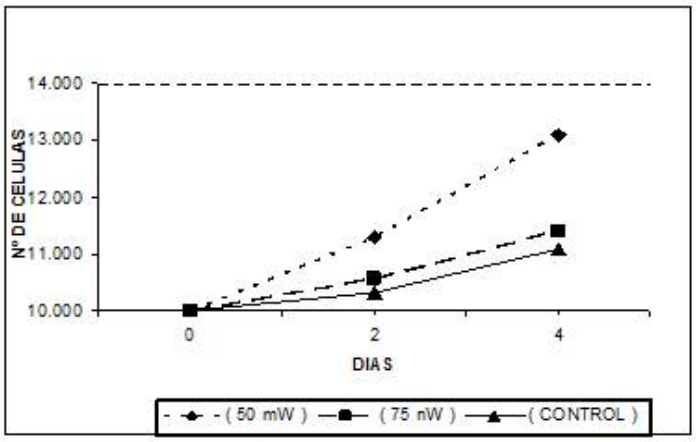
extracellular matrix, was evaluated using both transmission (TEM) electron microscopy. For TEM the samples were processed as follows: the pellet of chondrocytes were routinely processed for Durcapan® ACM (Fluka) resin-embedding. Then, ultra-thin sections (400 Å) were obtained, stained with 2% uranyl acetate and lead citrate, and examined and photographed with a ZEISS E. M. 109 (80 Kv) electron microscope, using a Kodak Eastman 5.302 film.

RESULTS

Cell growth kinetics.- The chondrocytes form secondary cultures progressively increased in number, increasing 10% spontaneously in the control at 4 days. In these cultures HPLP radiation at the low dose significantly increases the cell number (≈ 20%) whereas high dose was without effect (≈ 5%).

	Secondary cultures	**% variation**
Controls	10.000	
Controls 4 days	11.000	10%
50 MV	13.000	30%
75 MV	11.500	5%



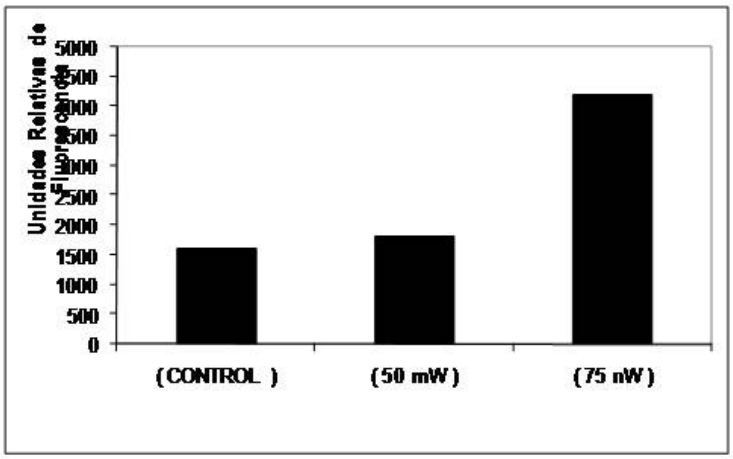


Flow cytometry.- The effects of culture procedures on the genetic background of chondrocytes was studied using flow cytometry at the ending of the experiments. Results demonstrate that all chondrocytes from secondary cultures were diploid, and were stopped at the G0/G1 phase of the cell cycle. The laser radiation did not modify any parameter and do not produce mutations in these cells (data not shown).

Cell apoptosis alter laser radaition.- In both controls and cells incubated in a medium free of caspase-3 substrate the fluorescence levels were similar and regarded as background. These values were substracted from the experimental values. In the secondary cultures radiated with 50 mW no significant increase in the casapase-3 activity was observed whereas after 75 mW radiation there was a 2.6-folds increase in the caspase-3 activity.

. **Secondary cultures**

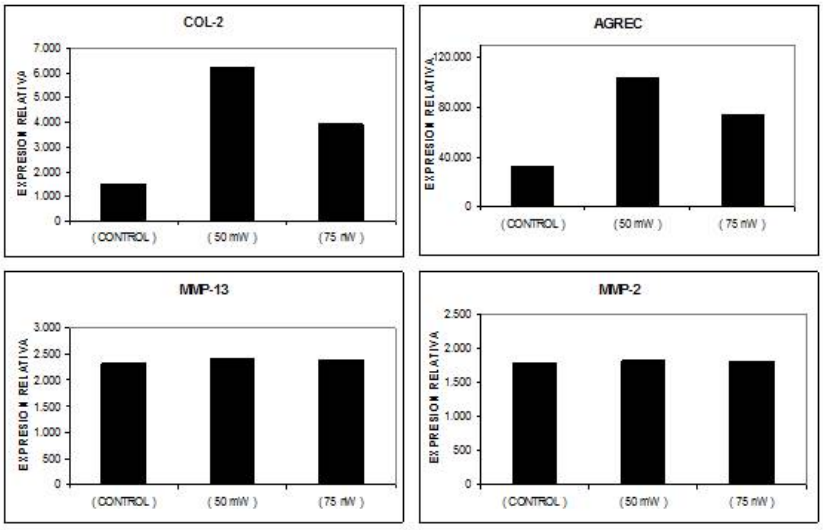
Controls	Residual	
50 mV	↑ 0.002	No increase of cell death
75 mV	↑ 2.6	Significant increase of cell death



Gene expression analysis.- The effect of HPLP radiation on the expression of some genes codifying for extracellular matrix components was also analyzed. The results were as follow:

Secondary cultures

	Col2	AGC1	MMP2	MMP13
Controls	Basal	Basal	Basal	Basal
50 mV	↑ 4.1	↑ 2.6	No changes	No changes
75 mV	↑ 3.2	↑ 2.3	No changes	No changes



Measurement of some extracellular matrix components in the culture medium, as determined by ELISA. Some of the extracellular matrix components are actively secreted to the culture medium. So, we have determined by ELISA the possible variation in the secretion of these molecules as a consequence of HPLP radiation.

	Col2	AGC1	Iα3	Iα1	Iα5	MMP2	MMP13
Controls	++	++	+	+	+	-	-
50 mV	++	+	+	+	-	-	-
75 mV		++ +		-	+ ++	-	-

Western-blot.- The results from the wester-blot experiments, carried out in triplicate, were as follows:

1.- Type II collagen. The type II collagen is the main fibrillar component of the extracellular matrix in the hyaline cartilage. It is the responsible for the functional architecture and biomechanical properties of AC.

Controls	Basal
50 mV	No changes
75 mV	No changes

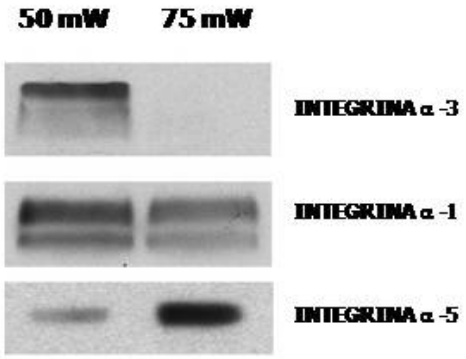
2.- Proteoglycans. They are the responsible for AC hydratation, retaining water within the tissue.

Controls	Basal
50 mV	Slight decrease
75 mV	Slight decrease

3.- Integrins. They represent one of the familias of adhesión molecules involved in the atachmet of the chondrocytes to the extracellular matriz and the different components of the extracellular matriz among them.

Controls	Residual
50 mV	No changes in α3 and α1, decrease of α5

75 mV	Loss of α3, no changes of α1 y increase of α5

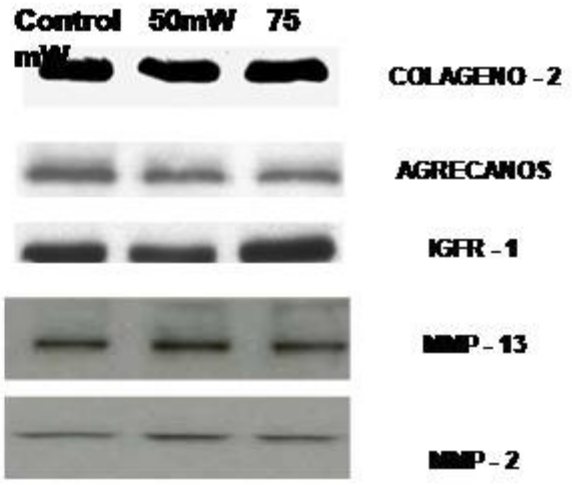


4.- IGF-R1. The biology of the chondrocytes in regulated at least partially by different growth factors, including the insulin-like growth factor family which play anabolic effect on AC. Here we have analyzed the effect of laser radiation on the expression of the type I-receptor for these factors.

Controls	Residual
50 mV	No changes
75 mV	Increase

5.- MMP-13 y MMP-2. The components of the extracellular matrix have a permanent tournover. In the degradation of these composunds are envolved a series of enzymes, especially MMP (matriz metalloproteases). In this study we analyzed two of them: MMP2 which degradates proteoglycans, and MMP13 which degradates type II collagen. HPLP radiation with independence of the dose did not change the expression of these molecules.

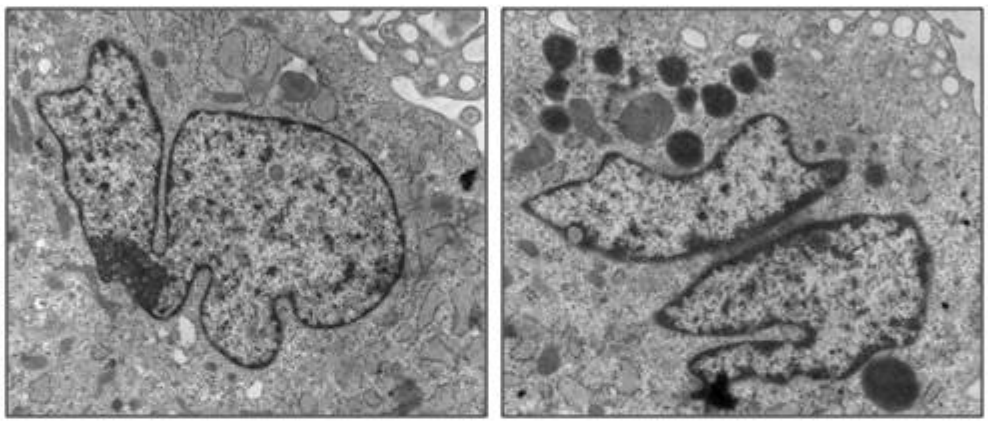
Controls	Residual
50 mW	No changes
75 mW	No changes



Inmunohistochemistry.- Results form the immunohistochemical study matches tose of the Western-blot and are summarized in the following table.

	Col2	AGC1	Iα3	Iα1	Iα5	IGFR1	MMP2	MMP13
Controls	++	++	+	+	+	++	-	-
50 mW		++ +		+	+	-	++ -	-
75 mW		++ +		-	+	++	++ -	-

Morfology of the chondrocytes and cultured matriz.- To analyze whether of not HPLP radiation is able to alter the morphology of the cultured chondrocytes we carried out an study using transmission electron microscopy. Radiated chondcrocytes were absolutely normal. The same is true for the extracellular matriz present in the medium.



CONCLUDING REMARKS

1.- HPLP radiation at a dose of 50 mW induces cell proliferation in secondary cultures of human chondrocytes, whereas a higher dose (75 mW) was without effect in this parameter. Probably this is due to an increase apoptotic cell death caused by high dose of HPLP.

2.- The HPLP radiation doses used in the present study were not mutagenic for secondary cultures of human chondrocytes.

3.- The HPLP radiation doses used increase the expressio, ath the mRNA and protein levels, of most of the components of the extracellular matrix (type II collagen, proteoglycans, IGFR1) although in some cases this is not paralelled by a secretion into the medium (proteoglycans).

4.- The HPLP radiation doses used here alter gene expression and synthesis of the integrins analyzed here. This suggest that HPLP radiated cells might have altered the ability to link the extracellular matrix, as well as of the extracellular matrix components among them. Nevertheless, it is impossible to affirm whether this change is due to an impaired synthesis or to a direct effect of HPLP on these molecules.

5.- The expression of the analyzed MMPs, i.e. MMP2 y MMP13, was no affected by the used HPLP doses.

Taken together the present results strongly suggest that HPLP radiation might be used in clinic to:

1.- Favour cell proliferation and extracellular matrix synthesis in auotologous chondrocytes transplantation.

2.- Favour the anabolic processes of AC in osteochondral pathologies; however, it is necessary to confirm that HPLP do no causes any structural alteration *in vivo*.

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