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LETTER TO THE EDITOR

EFFECTS OF GLUCOSAMINE AND NUCLEOTIDE ASSOCIATION ON FIBROBLAST: EXTRACELLULAR MATRIX GENE EXPRESSION

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Glucosamine (Gluc) is a drug used as an anti-inflammatory in moderate forms of knee arthrosis. A further off label use of Gluc is in the anti-aging treatments associated with Polideoxirybonucleotide (PDRN) through intra-dermal injection for a procedure called bio-stimulation. An unexpected effect on cultured dermal fibroblasts, during an experimental study on the gene activation in aesthetic bio-stimulation, was observed. The results have potential application in orthopaedic medical therapy. Fibroblast primary cultures were carried out, seeding cells on a layer of Gluc or PDRN alone or in combination for 24 h. Real Time-PCR was performed to investigate several gene expressions. The MMP13 and the IGF-I gene expression in fibroblast cultures were strongly inhibited after 24 h of incubation with the association of Gluc and PDRN, whereas Gluc and PDRN alone produced a modest inhibition of IGF-I and an activation of MMP13. MMP13 is present in osteoarthritic cartilage and this enzyme plays a significant role in cartilage collagen degradation. IGF1 is involved in growth and development and is successfully used in tissue-engineering for cartilage repair. Based on the reported data, we infer that the association of Gluc and PDRN has a potential application in cartilage therapy. Additional basic science and clinical studies are needed to confirm this preliminary report.

Glucosamine (Gluc) is an amino-sugar that is involved in glycosaminoglycan (GAG) synthesis. Acetic acid and glucosamine form N-acetylglucosamine that, after polymerization with glucuronic acid, produces hyaluronic acid (HA). HA derived from shellfish by hydrolysis is used in the therapy of moderate forms of knee arthrosis as an anti-inflammatory drug.

A further off-label use of Gluc is in anti-aging treatments where the association Gluc and Polideoxirybonucleotide (PDRN) is injected intra-dermally, such treatment is called bio-stimulation. The ratio of this therapy is to improve the production of HA in the dermal compartment without using its direct injection but through a mesotherapic administration of its precursor and to enhance cellular replication and protein synthesis in dermal fibroblasts with the simultaneous use of nucleotides (1).

Many objectives of aesthetic medicine are

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common to other medical branches. The role of HA in orthopaedics and its therapeutic use are among these.

This paper derives from the observation of an unexpected effect on cultured dermal fibroblasts during an experimental study on the gene activation in bio-stimulation aesthetic procedure. The obtained gene activation could have interesting application in orthopaedic medical therapy.

MATERIALS AND METHODS

Primary Human Dermal Fibroblast cell (HFb) culture

Fragments of dermal tissue of healthy volunteers were collected during surgery. The pieces were transferred into 75 cm^2 culture flasks containing DMEM medium (Sigma Aldrich, Inc., St Louis, Mo, USA) supplemented with 20% fetal calf serum, and antibiotics (Penicillin 100U/ml and Streptomycin 100 micrograms/ml-Sigma Aldrich, Inc., St Louis, Mo, USA). The cells were incubated in a humidified atmosphere of 5% CO2 at 37°C. Medium was changed the next day and twice a week thereafter. After 15 days the pieces of dermal tissue were removed from the culture flask. Cells were harvested after 24 days of incubation.

Cell culture

For the investigation, HFb at the second passage were seeded onto a layer of either glucosamine sulphate 400 mg (Dona, Rottafarm, Milan, Italy) or polideoxyribonucleotide 5.625 mg (Placentex Integro, Mastelli, Sanremo, Italy), or these two drugs combined. A set of untreated cells were used as controls. The cells were maintained in a humidified atmosphere of 5% CO2 at 37°C for 24 h. After the end of the exposure time, cells were trypsinized and lysed for RNA extraction.

RNA processing and Real Time PCR

Reverse transcription to cDNA was performed directly from cultured cell lysate using the TaqMan Gene Expression Cells-to-Ct Kit (Ambion Inc., Austin, TX, USA) following the manufacturer's instructions. Briefly, cultured cells were lysed with lysis buffer and RNA was released in this solution. Cell lysate were reverse transcribed to cDNA using the RT Enzyme Mix and appropriate RT buffer (Ambion Inc., Austin, TX, USA).

Finally the cDNA was amplified by real-time PCR. Amplification was performed by using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the specific assay designed for the investigated genes. SYBER assays reactions were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). Each reaction contained 10 µl 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 400 nM concentration of each primer, and cDNA.

All experiments were performed including non-template controls to exclude reagent contamination. PCRs were performed with two biological replicates. Expression was quantified using real time RT-PCR. The gene expression levels were normalized to the expression of the housekeeping gene TFRC. An evaluation of the expression as fold changes relative to the expression of the untreated HFb was performed. Quantification was made with the delta/ delta calculation method. Forward and reverse primers for the selected genes were designed using primer express software (Applied Biosystems, Foster City, CA, USA) and are listed in Table I.

RESULTS

Dermal fibroblast behaviour was evaluated by measuring the gene expression levels of several extracellular matrix related genes. Fig. 1 illustrates the effects of exposure to Gluc and to PDRN alone or combined.

DISCUSSION

In this experiment, COL11A1 gene expression was decreased after incubation of the single tested substances alone and combined. Collagen XI is known to belong to the fibrillar collagen group; the molecule is composed of three different chains each of that encoded by a different gene and is organized in cartilage fibrils with collagen II and IX. Its role is the regulation of fibril size. Moreover, it is connected to eparan and dermatan sulphate of extracellular matrix, contributing to its stabilization and in maintaining tissue integrity (2).

Further experiments are needed to explain the behaviour observed in this study. Metalloproteinases (MMPs) are known to be the major enzymes responsible for ECM degradation and tissue remodelling. The knowledge of the function of MMPs in biology and pathology is still limited but some of their biological activities seem paradoxical, in fact, they may exhibit pro- or anti-inflammatory effects, or pro- or anti-angiogenic activities.

Moreover, the role of growth factors and cytokines has increasingly become relevant in medicine and in orthopaedics for their effects on

Table I. *Primers sequences for SYBR® Green assay.*

Gene symbol	Gene name	Primer sequence (5'>3')
COL11A1	Homo sapiens collagen, type XI, alpha 1	F-AGATGAGGCAAACATCGTTGA R-ATCAGAATCCCTGCCGTCTA
IGF1	Homo sapiens insulin-like growth factor 1	F-GCGCAATGGAATAAAGTCCT R-ACAGCGCCAGGTAGAAGAGA
MMP2	Homo sapiens matrix metallopeptidase 2	F- TACGATGGAGGCGCTAATGG R- CGCATGGTCTCGATGGTATT
MMP3	Homo sapiens matrix metallopeptidase 3	F-TTTCCCAAGCAAATAGCTGAA R-AGTTCCCTTGAGTGTGACTCG
MMP13	Homo sapiens matrix metallopeptidase 13	F-AGTTCGGCCACTCCTTAGGT R-TGGTAATGGCATCAAGGGAT
DSP	Homo sapiens desmoplakin	F-ATGACCTGAGGAGAGGACGAA R-AGGCTCTCTCTTTCCTGTACCAC
ELANE	Homo sapiens elastase, neutrophil expressed	F- CTACGACCCCGTAAACTTGCT R- CCTCACGAGAGTGCAGACGTT

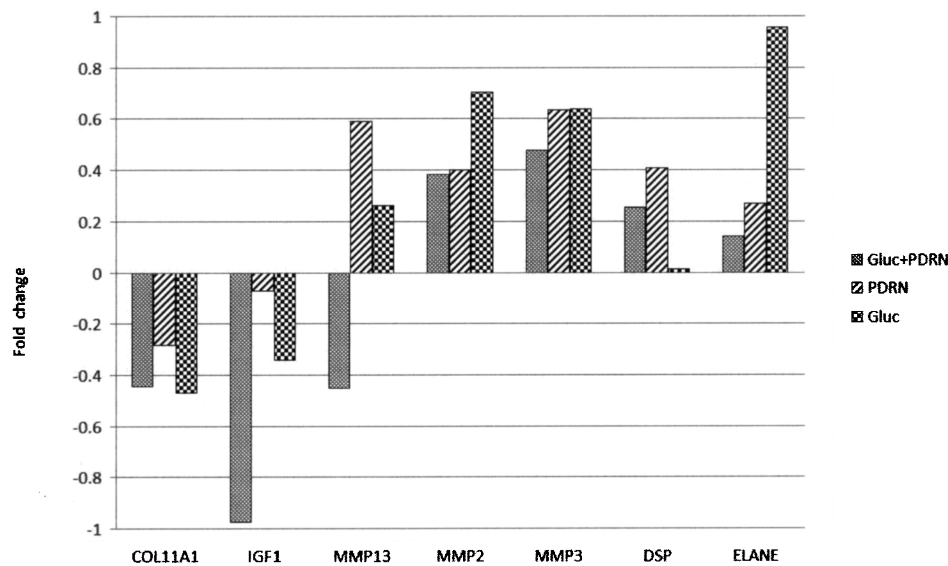

Fig. 1. *Effect of Gluc, PDRN and both drugs on primary human dermal fibroblast cell culture.*

articular cartilage homeostasis and in the study of the development of osteoarthritis and osteoarthritis-associated pain (3).

Twenty-three MMPs are known in humans, their expression is controlled by cytokines, growth factors, hormones, intercellular and cell–matrix interaction. They are also regulated by two major types of endogenous inhibitors: α2-macroglobulin and by tissue inhibitors of metalloproteinases (TIMPs) (4). MMP-13 is a collagenase responsible for cleavage of interstitial collagens I, II and III but can digest other ECM molecules and soluble proteins.

MMP-2 is a gelatinase, which digest a number

of ECM molecules including type IV, V and XI collagens, laminin, aggrecan core protein, etc. MMP-2 is active against collagens I, II and III in a similar manner to the collagenases

MMP-3, or stromelysine, has a domain arrangement similar to that of collagenases, but it does not cleave interstitial collagens. MMP-3 and MMP-9 have protective roles in atherogenesis (5).

The genes responsible for the three MMPs tested in this study are activated by Gluc and PDRN alone, and for MMP2 and 3 by the two drugs given in association, but in the last case MMP13 was strongly inhibited. MMP13 is known to have the effect of osteoclast activation by cleaving collagen I. Furthermore, Mitchell et al. observed the presence of MMP13 in osteoarthritic cartilage and that its activity against type II collagen indicates that the enzyme plays a significant role in cartilage collagen degradation (6). Thus, the negative synergism highlighted by this study could improve the research on a potential interesting therapeutic tool.

A similar effect is observed on Insulin-like growth factor 1 (IGF-I) gene expression that is slightly inhibited by Gluc or PDRN alone and is strongly down regulated by the association. The total amount of negative pick is greatly superior to the sum of the single values. Among the growth factors, IGF-I is known to stimulate the proliferation and the proteoglycan synthesis in cultured chondrocytes (7). IGF-1 presents structural and functional analogies to insulin and is involved in growth and development. IGF-1 is successfully used in tissue-engineering for cartilage repair and its homeostatic control was demonstrated; in fact, in cell cultures continuously stimulated by IGF-1 or TGF-β1 the massive growth factor leftovers in the environment down regulates the expression of the associated receptors (8).

Moreover, transgenic chondrocytes exhibited enhanced levels of IGF-I and IGF-II mRNA, and IGF-I and -II and IGF-binding proteins are known to be potent regulators of cartilage and bone growth. IGF-I and -II are known to stimulate proliferation and proteoglycan synthesis in cultured chondrocytes (8).

In osteoarthritic synovial fluid the IGF-1 level is increased and its overproduction is demonstrated by condrocytes in osteoarthritis. It can act both as paracrine and autocrine modulator to stimulate matrix synthesis and inhibit matrix degradation (9).

DSP is an obligate component of functional desmosomes and it encodes desmoplakin, an important protein in cell-cell junction maintenance and thus promotes epidermal integrity. Its importance in other compartments is outlined by different papers where the presence of DSP was demonstrated in cells derived not only from ectoderm but also from mesenchyme such as cardiac, corneal fibroblasts and odontoblasts (10-12).

A high abundance of DSP was identified in synovial fluid in osteoarthritis (10). In this study, DSP encoding gene was activated only in the samples where PDRN was present alone or associated with Gluc. This finding can support the idea of an ECM optimization performed by this therapy.

ELANE is the gene encoding for neutrophil elastase: the degrading elastin enzyme. Elastin is a structural protein that provides resilience to biological tissues. its role in conferring dermal elasticity and in damaged skin reparation and regeneration is well known. Elastin contains glycine, proline and multiple lysine-derived crosslinks which put together the individual polypeptide chains into a network (13). Elastin is initially synthesized as a soluble polypeptide, then the single molecules are aligned and stabilized by the formation of intermolecular bridges which contribute to its insolubility (14). Neutrophyl elastase is a serine protease of neutrophyl and monocyte granules (15) that has a role in connective tissue through MMPs activation (16). It was demonstrated that PMNs are able to down regulate the pro-inflammatory cytokine production of human monocytes through a soluble factor released from neutrophils. Elastase was found to be partially responsible for the observed effects. Furthermore, IL-1β and TNF-α can be degraded by elastase and neutrophil-derived proteases in PMN lysates. In two murine models, a zymosan-induced arthritis model and an endotoxemia model, the absence of PMNs *in vivo* results in aggravated arthritis and in increased cytokine levels, suggesting that the neutrophil can play a role in reducing the pro-inflammatory response both *in vitro* and *in vivo* (17).

In this study the ELANE gene was highly over-expressed after incubation with Gluc, less over-expressed with PDRN, but there was a synergistic effect with the association that could be useful to

reduce elastase activation given by Gluc.

In conclusion, the MMP 13 and the IGF-I gene expression in fibroblast cultures are strongly inhibited after 24 h of incubation with the association of Gluc and PDRN, whereas the Gluc and PDRN alone produced a modest inhibition of IGF-I and an activation of MMP13.

Based on the reported data we infer that the association of Gluc and PDRN has a potential application in cartilage therapy. It is our knowledge that additional basic science and clinical studies are needed to confirm this preliminary report.

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