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ENHANCEMENT OF FIBROBLAST PROLIFERATION, COLLAGEN BIOSYNTHESIS AND PRODUCTION OF GROWTH FACTORS AS A RESULT OF COMBINING SODIUM HYALURONATE AND AMINOACIDS

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Fibroblasts play a key role in tissue healing by producing the majority of extracellular matrix components, favouring granulation tissue formation, and stimulating re-epithelialization. Hyaluronan is a component of ECM and its anti-inflammatory effects and properties in enhancing wound closure are well known. In this study, we examined the effects of Aminogam® gel, a new pharmacological preparation suggested to improve wound healing, composed of hyaluronic acid, proline, lysine, glycine and leucine, on human fibroblasts. Results show that fibroblasts treated with hyaluronic acid plus aminoacid solution increased their proliferative activity, collagen I and III, and fibronectin synthesis. Moreover, HA plus aminoacid solution increased the expression of transforming growth factor beta, connective tissue growth factor, interleukin-6 and -8, assayed by RT-PCR. These results suggested that Aminogam® gel, involved in several stages of wound healing, as fibroblast proliferation, granulation tissue formation, ECM component deposition, and production of cytokines, may be a useful device to favour and accelerate wound closure.

Wound healing is characterized by different steps involving generation of new vessels, fibroblast proliferation and differentiation, deposition of extracellular matrix (ECM) and re-epithelialization. Fibroblasts play a key role in this process, as wound closure requires fibroblast proliferation and migration into granulation tissue, followed by sequential depositions of specific ECM components, wound contraction and remodelling. In fact, fibroblasts promote neo-angiogenesis, secrete all the components of the ECM (glycosaminoglycans, proteoglycans, glycoproteins and collagens) and produce several cytokines and growth factors (1).

Hyaluronic acid (hyaluronan, HA), a glycosamminoglycan, is the major component of pericellular matrix, where it plays multiple complex roles in cell adhesion, proliferation and locomotion. In physiological conditions, the most widely distributed form of HA is a high molecular weight (HMW) polysaccharide, while low molecular weight (LMW) polysaccharides derive from *de novo* synthesis or from degradation of HMW HA by hyaluronidases

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and free oxygen radicals and are more abundant in inflammatory processes (2). Even though both HMW and LMW polymers interact with membrane receptors, such as CD44, RHAMM, LYVE-1 and layilin (3-5), they have different proliferative activities, probably depending on hyaluronan/versican ratio in pericellular matrix (6) and on cell type involved (7-8). A variety of HA-induced cellular responses, including proliferation, migration and cytokine synthesis are mediated by CD44, which is expressed on cell surface; its binding to HA depends on expression levels, on type of CD44 isoform, and on glycosylation status (9). Moreover, hyaluronan is involved in assembly of ECM proteins, where it serves as a scaffold for matrix proteins (10), promotes recovery from tissue injury, and exerts an anti-inflammatory activity in osteoarthritis and other joint diseases (11). Hyaluronan is a highly hygroscopic molecule and this property is believed to be important for modulating tissue hydration and osmotic balance. Finally, hyaluronan may facilitate collagen reorganization and contraction during repair (12).

Collagen deposition is essential in wound healing. The biosynthesis of collagen proteins includes formation of procollagen chains and successive proline and lysine residue hydroxylation. These intracellular enzymatic modifications of proline and lysine residues occur before triple helix formation, and hydroxyproline or hydroxylysine are critical for ultimate stabilization of the triple helical procollagen structure (13-14). Once in the extracellular environment, further modifications occur that ultimately lead to deposition and cross-linking required for normal ECM formation (15). Availability of a pool of aminoacids (AA), especially those involved in collagen synthesis, may improve and accelerate wound closure.

Aminogam® is a new medical device, in form of gel preparation, composed of sodium hyaluronate and four aminoacids (glycine, leucine, proline, and lysine) involved in wound healing (16).

The aim of this study, therefore, is to investigate the biological effects of Aminogam® constituents on human neonatal fibroblasts MRC-5, by evaluating cell proliferation, cell cycle analysis and, by RT-PCR, the expression profiles of collagen I and III, two of the major ECM components, and of fibronectin, acting as a scaffold for the deposition of several proteins. Moreover, the expression profiles of two genes related to the production of ECM proteins, such as transforming growth factor beta (TGF β) and connective tissue growth factor (CTGF) were evaluated. Finally, the expression levels of two inflammatory cytokines, namely interleukin-6 and -8 (IL-6 and IL-8) were analyzed.

MATERIALS AND METHODS

Hyaluronic acid plus aminoacid solutions

Aminogam® gel preparation contains 1.33% sodium hyaluronate (pm 1.64 x 10^6 Da) and 2% aminoacid pool (respectively 0.1% lysine HCl, 0.75% proline, 0.15% leucine and 1% glycine). Biological effects of Aminogam® on cell cultures were tested utilizing H_2Od solutions with HA and aminoacids (sterile powders were provided from Errekappa Euroterapici, Milan, Italy) at the same ratio of gel preparation. A mother solution, containing 0.665 % HA and 1% AA, was serially diluted in distilled water.

Cell culture

Human foetal lung fibroblasts (MRC-5) were cultured in MEM (Minimum Essential Medium with Earle's salts; Euroclone, Ltd.) supplemented with 10% foetal calf serum, 100 µg /ml penicillin, 250 µg /ml streptomycin and 2 mmol L-glutamine (Euroclone, Ltd). MRC-5 cells were utilized between the 25th and 28th passages. Cell line was cultured at 37°C in a humidified atmosphere of 5% CO_2.

Proliferation assay

Cells ($3x10^3$) in 90 µl of appropriate complete medium were plated into 96-well microtiter plates for 24 h. HA and aminoacids were freshly dissolved in water and administered in 10-fold concentration (10 µl) for a minimum of six serial dilutions, in triplicate. Three days after, medium containing HA plus AA solution was gently aspirated from each well and replaced with fresh complete MEM. Then, an aliquot of 10 µl of 3-(4,5 dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) solution (5 mg/ml H_2Od) was added to each well and plates were incubated for 4 h at 37°C. To achieve the complete solubilization of formazan crystals, 100 µl of a solution of 10% SDS in 0.01 M HCl was added to each well and plates were incubated at 37°C for 24 h. The absorbance was measured on a microplate reader (model 680, Bio-Rad Laboratories) at 570 nm. Results were obtained by 5-6 independent experiments and were expressed as media ± standard deviation (SD).

Cell cycle analysis

To analyze the effects of HA plus aminoacids on

the cell cycle, cells (plated as 3×10^5 in 9 ml of complete medium) were treated with 1 ml of solution containing 0.125% AA + 0.083% HA and incubated for different times. Cells were then harvested, washed twice with cold PBS and fixed in 70% ethyl alcohol at -20°C overnight. After fixation, cells were centrifuged, washed with PBS, and incubated at room temperature for 20 min with propidium iodide (PI) staining solution (50 µg/ml PI, 0.05% Triton X-100, 20 mg/ml RNase A in PBS). Cells were subjected to flow cytometric analysis of DNA content using flow cytometer (Coulter Epics XL-MCL) and data were analyzed by Mod Fit LT software (Verity Software House, Topsham, ME).

RT-PCR

MRC-5 cells (5 $\times10^5$) were plated in 25 cm^2 flasks and cultured for three days. After medium substitution, a solution containing 0.125% aminoacids (glycine, leucine, proline, lysine) and 0.083% sodium hyaluronate, in H_2Od was added (final concentration 0.0125% AA plus 0.0083% HA). Three and four days after starting the assay, cells were harvested, washed twice in PBS and counted. Total RNA was isolated from 5×10^5 cells by TRIzol reagent (Invitrogen), and spectrophotometrically measured. 2 ng of total RNA primed with oligo (dT), incubated at 42°C for 45 min, were used to synthesize c-DNA by SuperScript III First-strand Synthesis System for RT-PCR (Invitrogen, USA). Reverse transcription was terminated by heating at 70°C for 10 min and 1 µl of c-DNA was used as the template for PCR. Primers, annealing temperatures and number of PCR cycles are shown in Table I. GAPDH was used as housekeeping gene. The PCR products were analyzed on 2% agarose gel stained by ethidium bromide. Image acquisition was performed by UV trans-illumination system (Gel Doc 2000, Quantity One Software, Bio-Rad Laboratories).

Statistical analysis

Unpaired Student's *t* test (p value according to Bonferroni) was used for statistical analysis of proliferation assay data.

RESULTS

The combination of hyaluronan and aminoacids enhanced MRC-5 proliferation (Fig. 1). This effect was visible by MTT assay after three and four days of incubation and, in both cases, cell growth induced by HA plus AA. was statistically significant *vs* untreated cells from concentration of 0.0041% HA + 0.0062 AA to concentration of 0.0332% HA + 0.05% AA, while higher HA plus aminoacid amounts gave a not quite statistically significant result. Sodium HA has high molecular weight and its concentrated solutions have high osmolarity which can damage cells and mask proliferative effect *in vitro*. Four days incubation was less effective in stimulating fibroblast growth than three days, probably due to hyaluronan and/or aminoacid degradation in culture medium.

The effects of hyaluronan and aminoacids on cell cycle are shown in Fig. 2. MRC-5 cells have a doubling time of about 2 days. Cell exposition to drug solution led to acceleration of progression

Table I. *Primer sequences and PCR condition for cDNA amplification.*

mRNA	Primers	Annealing temperature	Number of cycles
COLLAGEN I	For 5'-CTGGCAAAGAAGGCGGCAAA-3' Rev 5'-CTCACCACGATCACCACTCT-3'	60°C	25
COLLAGEN III	For 5'-GATATTGCACCCTATGACATTG-3' Rev 5'-GTTGAAGTTTATTTATTATAGCACC-3'	60°C	30
FIBRONECTIN	For 5'-GCCTGGTACAGAATATGTAGTG-3' Rev 5'-ATCCCAGTGATCAGTAGGCTGGTG-3'	60°C	25
IL-6	For 5'-ATGAACTCCTTCTCCACAAGCGC-3' Rev 5'-GAAGAGCCCTCAGGCTGGACTG-3'	60°C	30
IL-8	For 5'-ATGACTTCCAAGCTGGCCGTGGCT-3' Rev 5'-ATGACTTCCAAGCTGGCCGTGGCT-3'	60°C	30
TGF β	For 5'-AACACATCAGAGCTCCGAGAA-3' Rev 5'-GTCAATGTACAGCTGCCGCAC-3'	60°C	28
CTGF β	For 5'-CGAGCTAAATTCTGTGGAGT-3' Rev 5'-CCATGTCTCCGTACATCTTC-3'	56.5°C	28
GAPDH	For 5'-TTGGTATCGTGGAAGGACTCA-3' Rev 5'-TGTCATCATATTTGGCAGGTTT-3'	55°C	30

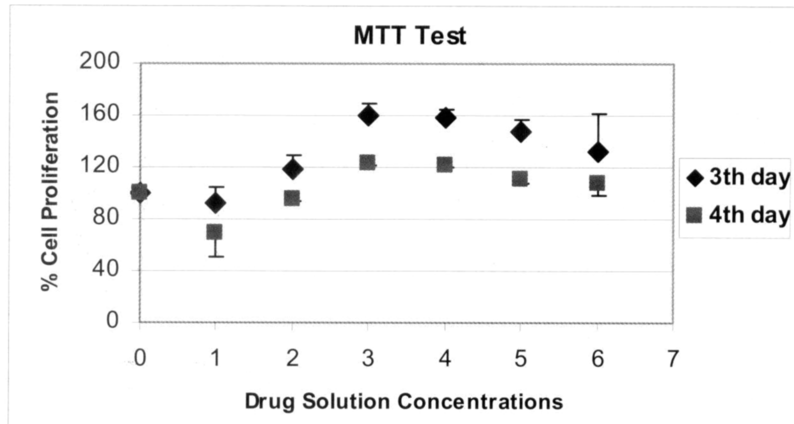

Fig. 1. *Proliferative activity of HA + AA on human fibroblasts as evaluated by MTT assay. Final drug solution concentrations: 1= 0.002% HA + 0.0031 AA; 2= 0.0041% HA + 0.0062 AA; 3 = 0.0083% HA + 0.0125 AA; 4 = 0.0166% HA + 0.025% AA; 5 = 0.0332% HA + 0.05% AA; 6 = 0.0664% HA + 0.1% AA. Each point represents the mean ± standard deviation (SD) of 5-6 independent experiments.*

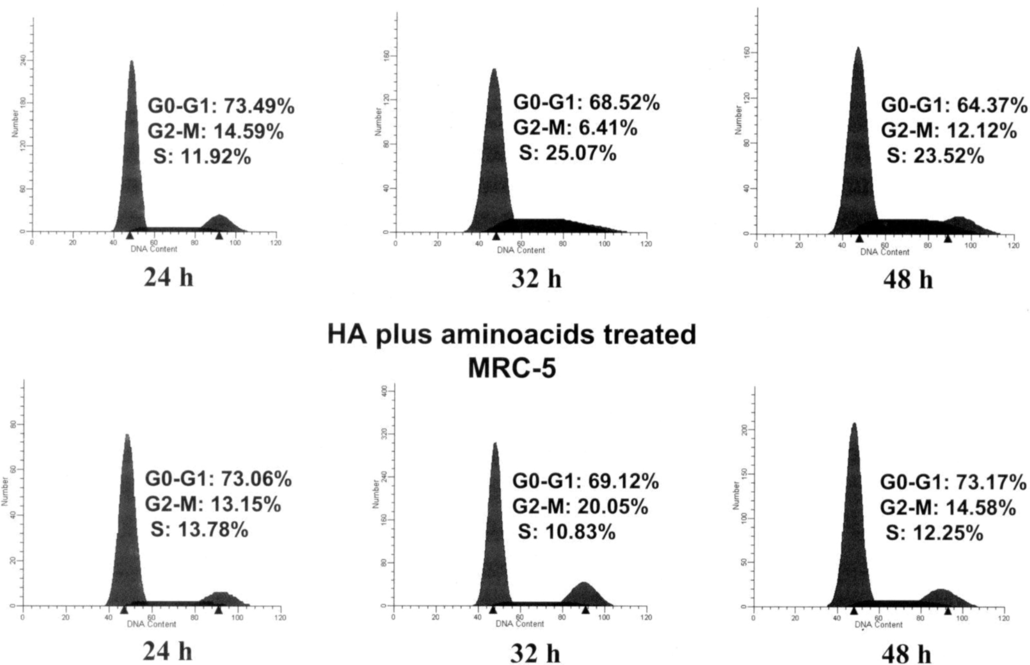

Fig. 2. *Cell cycle analysis by flow cytometry of MRC-5 cells treated with 0.0125 % AA plus 0.0083% HA (final concentrations) versus untreated cells. Cells were examined after incubation times of 24, 32 and 48 h. A representative experiment was shown.*

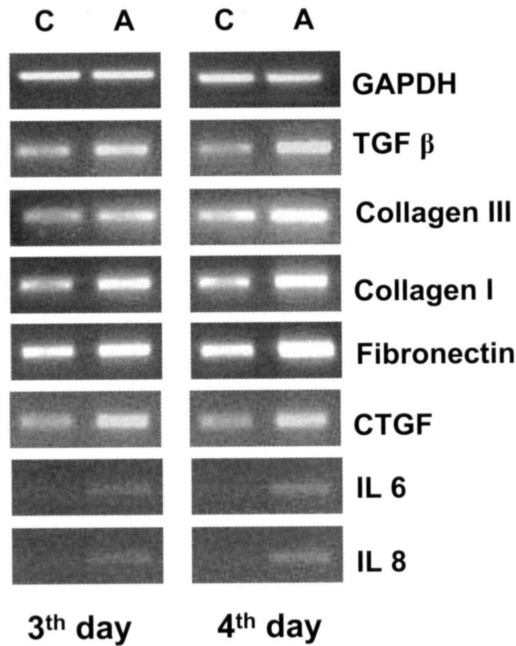

Fig. 3. *mRNA expression (RT-PCR) of some genes involved in wound healing in MRC-5 treated (A) and untreated (C) cells with a solution of 0.0125% AA plus 0.0083% HA. Determinations was performed after three and four days of incubation. GAPDH was used as housekeeping gene.*

from S to G2-M phase, especially after 32 h, and to the passage from G2-M to G0-G1 after 48 hours of incubation in comparison with untreated cells. This acceleration of progression in cell cycle phases explained the increase of cell number and confirmed cell proliferation results obtained with MTT test.

RT-PCR (Fig. 3) carried out on RNA extracted from treated fibroblasts evidenced an increased expression of fibronectin, collagen I and III, three proteins abundant in extra-cellular matrix. Expression levels increased after the third incubation day and reached maximum levels after the fourth day. The same expression profiles were seen for TGF β, while CTGF transcription increased on the third day and remained unchanged on the fourth day. IL-6 and IL-8, which were contained at very low basal levels in untreated cells, showed an increase after three and, particularly, four days of treatment.

DISCUSSION

In this work we tested some biological activities of Aminogam® gel, a preparation containing HMW hyaluronic acid plus four aminoacids, on human fibroblasts. While HA is a common component of pharmacological preparations for accelerating wound healing or to facilitating closure in chronic dermal wound, this is the first example, to our knowledge, of the presence of lysine, proline, leucine and glycine, four aminoacids involved in collagen synthesis and stabilization in a medical gel compound. We show that after treatment with Aminogam®, collagen I, collagen III and fibronectin production by fibroblasts increased.

Fibronectin is one of the most important provisional ECM glycoproteins: it promotes the migration and the adhesion of fibroblasts to the ECM (17), plays a role in adjustment of collagen fibrils, provides support for capillaries and acts as a repository of growth factors (18).

Collagens, especially collagen I representing about 80-90%, and collagen III, constituting the remaining 10-20% of dermal collagen (19), provide increased strength to the wound and facilitate macrophages and endothelial cell migration. All collagens consist of three polypeptide chains, termed α chains, that are characterized by repeating glycine-X-Y sequences. Position X is often occupied by proline or lysine and position Y by hydroxyproline or hydroxylysine. Glycine is required at every third position to allow the close packing of α chains within the triple helix (20). So, glycine (accounting for half content of Aminogam® aminoacid pool) constitutes one-third of the collagen molecule, while proline/hydroxyproline or lysine/hydroxylysine may represent about 23% of collagen chains (21).

Leucine residues are important constituents of the small leucine-rich repeat proteoglycans and proteins (SRLPs) that participate in the organization of the ECM and have important effects on cell behaviour. The best-characterized SLRP is decorin, thus named for its "decorating" association with collagen fibrils. Decorin controls the morphology of collagen fibrils and its absence results in non-uniform fibril thickness and skin fragility (22).

Deposition of newly synthesized collagen necessary for wound closure may be enhanced by

availability of aminoacids. For example, in wound fluid, proline concentration is 50% higher than in plasma (21). In the ECM, proline is provided by prolidase, a cytosolic enzyme that splits imidodipeptides with C-terminal proline (23) and that may be a rate-limiting factor in collagen production (24). Providing the diet with additional proline to enhance its bioavailability for collagen biosynthesis does not result in increased collagen accumulation, while arginine and ornitine supplementation seems to be effective in collagen deposition (21). Aminogam® gel is one of the few attempts to favour the delivery of aminoacids directly to the site of the wound. Not only aminoacids, but hyaluronan also can improve fibroblast production of collagens. This hyaluronan effect can be due to modulation of IL-1-induced inhibition of collagen synthesis by restoring insulin-like growth factor (IGF)-IR signaling cascade (25). This pathway is activated by IGF-I, recognized as the most potent stimulator of collagen biosynthesis in fibroblasts (26).

Fibroblasts growth and recruitment are critical to wound healing because they produce growth factors for intercellular signalling and proteins involved in remodelling of the clot and extracellular matrix. HA effects on cell proliferation are controversial. Recently, Kothapalli et al. reported HMW HA inhibited cell growth in vascular smooth muscle cells and in fibroblast by reducing induction of cyclin D1 (7), while previous reports indicated HA promoted osteoblast proliferation and differentiation (8). Furthermore, HMW hyaluronan supports tumour cell proliferation, progression and dissemination (27). Instead, few doubts regard proliferative activities of LMW HA, documented on smooth muscular (28), endothelial (29) and on cancer cells (30).

Our study demonstrates that treatment with HMW HA and aminoacids stimulates fibroblast proliferation. To discriminate between HA and aminoacid proliferative effect, we treated fibroblasts with the same concentrations of HA or aminoacids alone, as reported above (Fig. 1). Both Aminogam® components demonstrated proliferative activity (data not shown), but we cannot exclude fibroblast secretion of hyaluronidases with subsequent HMW HA enzymatic cleavage and production of small HA polymers which, in turn, may stimulate proliferation. However, a recent report underlines fibroblast proliferation following treatment with both native (HMW) and fragmented HA (31).

During the proliferative phase of healing, fibroblasts produce cytokines and growth factors, such as TGF-β, CTGF, IL-6 and IL-8. TGF-β is a pleiotrophic growth factor synthesized by different cell types and existing in three isoforms. Platelets and macrophages are the major source of TGF-β in wound healing (32). TGF-β stimulates fibroblast migration and proliferation (33), promotes matrix metalloproteinase expression to favour fibroblasts overcoming cellular debris (34) and collagen production (33) and, together with vascular endothelial growth factor (VEGF), enhance neo-angiogenesis (35). Furthermore, TGF-β stimulates re-epithelialization and phenotypic transformation of fibroblasts into myo-fibroblasts (33).

Connective tissue growth factor (CTGF) promotes migration and proliferation of fibroblasts into the wound (33, 36) and collagen deposition (37). In fibroblasts treated with HA plus aminoacids our results show an increased expression of both TGF-β and CTGF determining an autocrine and paracrine cellular stimulation, responsible in turn for an enhanced collagen deposition and neo-angiogenesis. We have previously demonstrated that Aminogam® was responsible for an over-expression of vascular endothelial growth factor (VEGF) by fibroblasts and promoted angiogenesis *in vivo* (16).

Finally, hyaluronan plus aminoacid treatment increased the transcription of IL-8 and IL-6 mRNA, the constitutive expression of which in fibroblasts is very low. These two cytokines carry out several functions in inflammation, bone resorption and osteoclast formation, and are fundamental in initiating the process of wound healing. In fact, IL-8 attracts to the wound site polymorphonuclear cells (PMNs) and macrophages which clean the inflammation site by removing cellular debris and bacteria and release several pro-inflammatory cytokines, out of all IL-6 which is also involved in keratinocyte proliferation and wound closure (33). The significant role for IL-6 in tissue repair was demonstrated by experiments in which IL-6$^{-/-}$ knockout mice had significantly delayed cutaneous wound healing and worse outcome (38).

In conclusion, this study documented multiple effects exerted by association of HMW hyaluronan

plus lysine, proline, leucine and glycine on human fibroblasts and suggests that this association may accelerate wound repair process from the early inflammatory stages to the final tissue recovery and that it may be a useful device in the treatment of chronic skin wounds, such as pressure and diabetic ulcers, the healing process of which is altered by not fully understood defects.

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