Using Hyaluronic Acid to Create a Fetal-like Environment in vitro

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The fetal wound healing matrix is exceptionally rich in hyaluronic acid (HA). Fetal wounds heal without scarring or contraction. Noting these observations, we cultured adult dermal explants in the presence of various concentrations of medical-grade HA in vitro. In the presence of HA, fibroblasts migrated from the dermal explant and multiplied more rapidly than control explants. Subsequently, sterile toothpicks were used to disrupt (wound) fibroblast monolayers mechanically and the rate of closure was monitored. Cells cultivated in the presence of 5 mg/ml of exogenous HA changed in morphology and closed the wound more quickly than control cultures. Cells surrounding the wound extended numerous podalic processes and showed increased interdigitation. The effect of HA on cell proliferation is usually discussed in terms of the mechanical effects HA exerts on cells and the extracellular matrix. The physiological effect of HA may lie in its ability to act as an accessory receptor in cooperative ligand-binding pathways. For example, HA may bind growth and/or other factors, and thereby increase the effective concentration of these factors at the cell surface.

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The fetal response to injury is fundamentally distinct from that of the adult. Fetal wounds heal without scarring or contraction. An elevated deposition of glycosaminoglycan hyaluronic acid (HA) has been observed in the extracellular matrix (ECM) of fetal wounds [1, 2]. HA is associated with tissue regeneration and repair [1], and may inhibit cytodifferentiation [2].

HA provides a favorable environment for cell growth and motility that may foster tissue regeneration instead of fibrosis [2]. This observation may have therapeutic implications in that HA application may modulate the healing of adult wounds in a manner more similar to the healing of fetal wounds [2].

HA is a highly conserved glycosaminoglycan that acts as a hydrating vehicle in a variety of vertebrate soft tissues. In vivo HA exists as sodium hyaluronate, which functions to impart stiffness, resiliency, and lubricious faculty to various tissues by nature of its propensity to form viscous hydrated matrices. This property of HA stems from its tendency to occupy 1,000 to 10,000 times its own volume when fully hydrated. The unique biophysical properties of HA are manifested in its mechanical roles in synovial fluid, vitreous humor, and the ability of connective tissues to resist compressive forces.

Highly purified medical-grade HA, extracted from the cell walls of select Streptococcus strains, is currently employed to reduce the incidence of postoperative adhesions, as a viscoelastic agent in intraocular surgery, a synovial replacement device, and in various cosmetic applications [3].

The biophysical properties of HA are augmented by the profound influence HA has on migrating and proliferating cells both in vivo and in vitro. HA is a major constituent of the ECM surrounding rapidly dividing and migrating cells, including embryonic mesenchyme [4], highly metastatic tumor cells [5], hematopoietic cells [6], rapidly dividing epithelial cells [7], and fibroblasts [8]. As a ligand for the cell surface receptor CD44, HA promotes fibroblast adhesion and migration [9], which is associated with transmembrane signaling via protein kinase modulation [10]. Proliferating cells avoid inhibitory cell—cell contacts by the deposition of pericellular HA. On deposition, HA, by nature of its hygroscopicity, expands the ECM, thereby maintaining intercellular distance, which facilitates the cell detachment and rounding requisite for mitosis [11].

The qualities of HA delineated earlier, in conjunction with recent advances in its manufacture [3] and the absence of untoward affects in humans [12], creates an opportune environment for

further research and development of biomedical products based on HA.

Therefore, we have studied the in vitro response of human dermis to a matrix based on highly purified, medical-grade HA. We specify the quality of the HA because some inconsistencies in the literature may stem from the use of HA with vastly different properties. In the present study, the response of human dermal explants and mechanically disrupted fibroblast monolayers to HA matrices was compared to controls maintained on conventional cell culture substrata.

Materials and Methods

Dermal Explants

Dermal biopsies were obtained from a 72-yearold male patient and were transported to cell culture facilities at Florida Atlantic University. Samples were minced into 2-mm cubes, observed at x20 to ensure uniformity, and plated 1/well in a 24-well culture plate (Falcon, Becton Dickinson Co, Lincoln Park, NJ) that had been coated with fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO). After the tissue fragments were allowed to adhere to the substrate for 1 hour, 1 ml of cell culture medium was added to each well. The culture medium consisted of Opti-MEM (Grand Island Biological Co., Grand Island, NY) supplemented with 10% FBS containing 100 µg /ml kenamycin (Sigma). Various concentrations of HA (10 mg /ml, 5 mg /ml, and 2.5 mg /ml with a molecular weight of 1.7 x 10^6 daltons and an intrinsic viscosity of 27 dL /g (Genzyme, Cambridge, MA) were added to comprise the experimental media. Control explants were cultured without exogenous HA. Cultures were incubated in a humidified (5% CO^2 and 95% air atmosphere) at 37°C. Each concentration of HA was tested in triplicate and the experiment was replicated three times.

After 5 days, growth was documented photographically in situ without fixatives or staining. Pictures were taken at x 100 (represented herein as x420) employing an inverted lens phase-contrast microscope, fitted with a Nikon 35-mm camera (Nippon Kogaku Inc, Garden City, NY), using Kodak Pan x-125 black-and-white film (Eastman Kodak Co, Rochester, NY).

In Vitro Wounds/Mechanically Disrupted Monolayers

In vitro wounds were created in a confluent layer of human fibroblasts based on the procedure reported by Longaker and associates [13]. Briefly, human fibroblasts (isolated from the biopsy material described earlier) at passage 6 were seeded onto glass coverslips (Corning Glass Works, Corning, NY) at 2.5 x 10^4 cells /coverslip. The coverslips were placed in wells of six-well culture plates, and 3 ml of medium was added per well. The medium used here consisted of Opti-MEM supplemented with 10% adult equine serum (AES), and 100 ,u,g /m1 kanamycin and 5 mg /ml HA. After 2 days of growth, the monolayers were mechanically disrupted with a sterile toothpick to create an X pattern. Control monolayers were scratched and cultured in media as described earlier, save HA. Each concentration of HA was tested in triplicate and the experiment was replicated three times. The coverslips were photographed at 15 minutes, 4 hours, 12 hours, and 24 hours postdisruption.

Results

Figure 1A represents the growth of a human dermal biopsy without exogenous HA at 5 days. Fibroblastic and epithelial cells are shown in a state of growth not uncommon for this early stage of culture. In comparison, Figures **113 —D** show similar biopsies grown in the presence of 10 mg /ml, 5 mg /ml, and 2.5 mg /ml, respectively, of exogenously added HA. The best growth was seen at 5 mg /ml of HA.

Figure 2A shows the in vitro scar, 15 minutes postwounding, created by scratching a confluent monolayer of fibroblasts grown on a glass coverslip. Figures 2B and C show the state of wound healing after 24 hours of growth in the absence and presence, respectively, of HA (5 mg /ml).

Discussion

The fetal healing rate, absence of scar tissue in healed fetal wounds, and the reversion to the "fetal state" by many cancerous cells all have elevated amounts of pericellular HA in common.

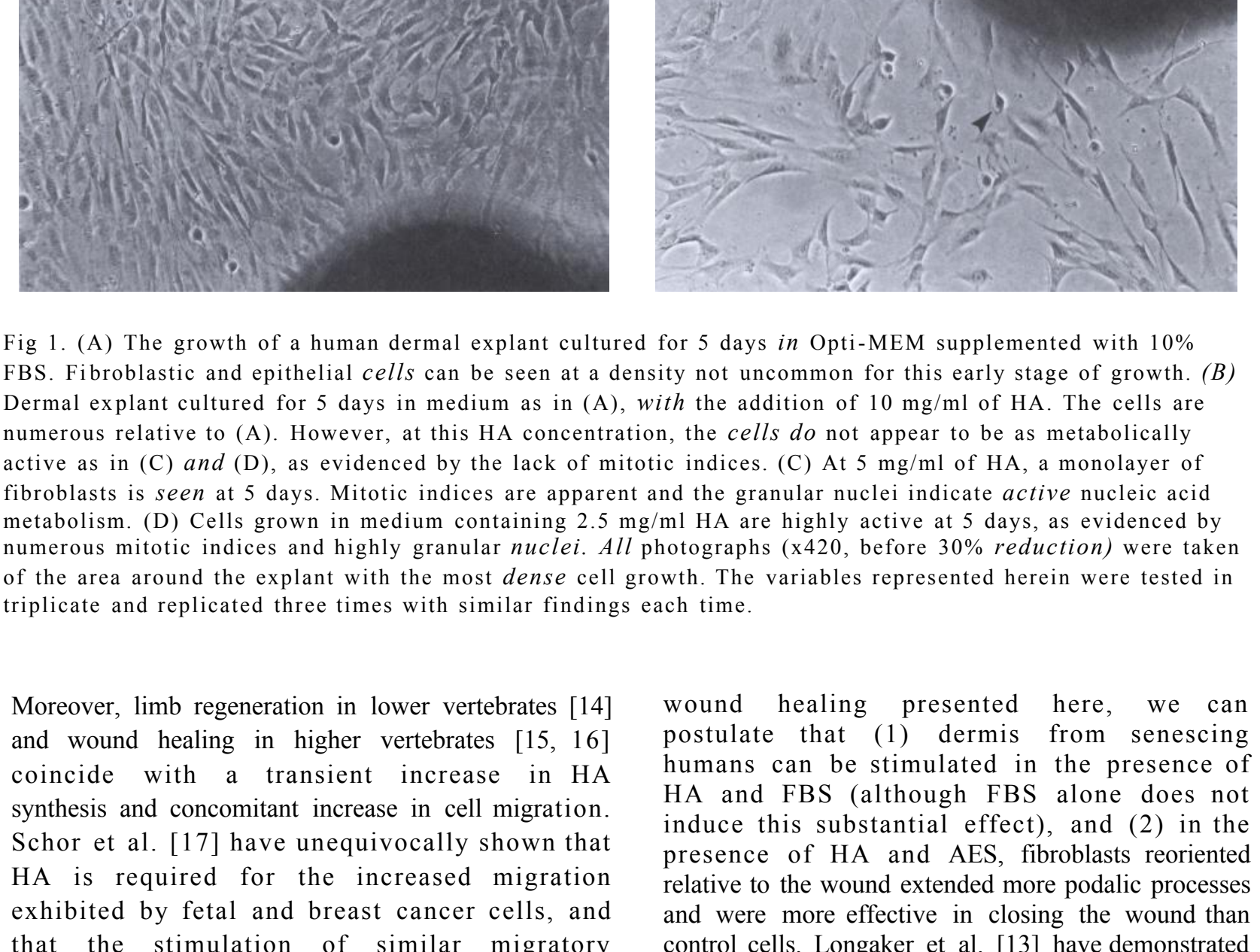

Fig 1. (A) The growth of a human dermal explant cultured for 5 days *in* Opti-MEM supplemented with 10% FBS. Fibroblastic and epithelial *cells* can be seen at a density not uncommon for this early stage of growth. *(B)* Dermal explant cultured for 5 days in medium as in (A), *with* the addition of 10 mg/ml of HA. The cells are numerous relative to (A). However, at this HA concentration, the *cells do* not appear to be as metabolically active as in (C) *and* (D), as evidenced by the lack of mitotic indices. (C) At 5 mg/ml of HA, a monolayer of fibroblasts is *seen* at 5 days. Mitotic indices are apparent and the granular nuclei indicate *active* nucleic acid metabolism. (D) Cells grown in medium containing 2.5 mg/ml HA are highly active at 5 days, as evidenced by numerous mitotic indices and highly granular *nuclei. All* photographs (x420, before 30% *reduction)* were taken of the area around the explant with the most *dense* cell growth. The variables represented herein were tested in triplicate and replicated three times with similar findings each time.

Moreover, limb regeneration in lower vertebrates [14] and wound healing in higher vertebrates [15, 16] coincide with a transient increase in HA synthesis and concomitant increase in cell migration. Schor et al. [17] have unequivocally shown that HA is required for the increased migration exhibited by fetal and breast cancer cells, and that the stimulation of similar migratory behavior in adult cells is dependent on the presence of HA. In the presence of hyaluronidase, the increased migration is abolished [17]. Hitzeman and colleagues [18] have demonstrated an increasing replication potential, in low-density cell cultures, concomitant with HA deposition.

Considering the dramatic results of the dermal explant cultures and the improved in vitro wound healing presented here, we can postulate that (1) dermis from senescing humans can be stimulated in the presence of HA and FBS (although FBS alone does not induce this substantial effect), and (2) in the presence of HA and AES, fibroblasts reoriented relative to the wound extended more podalic processes and were more effective in closing the wound than control cells. Longaker et al. [13] have demonstrated that the positive effects of fetal sera on wound healing may reflect the amount of HA synthesis induced by components of fetal sera. Our study shows that the closure of a mechanical disruption (in vitro wound) of this type is accelerated by the addition of exogenous HA in the presence of adult serum.

These findings suggest that large amounts of

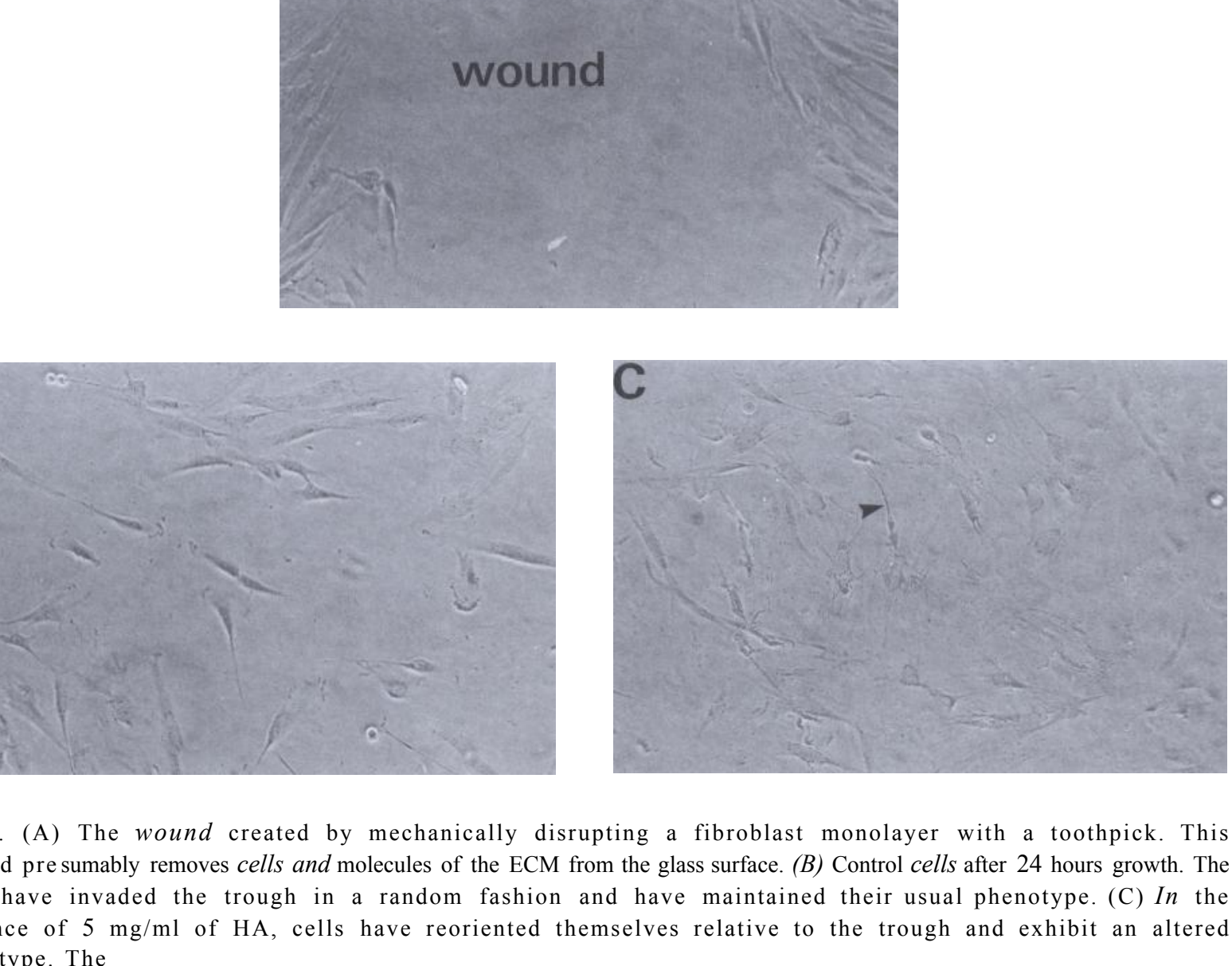

Fig 2. (A) The *wound* created by mechanically disrupting a fibroblast monolayer with a toothpick. This method presumably removes *cells and* molecules of the ECM from the glass surface. *(B)* Control *cells* after 24 hours growth. The cells have invaded the trough in a random fashion and have maintained their usual phenotype. (C) *In* the presence of 5 mg/ml of HA, cells have reoriented themselves relative to the trough and exhibit an altered phenotype. The changes seen here are reminiscent of the transformation of normal fibroblasts to the myoblastic phenotype, *which* is characteristic of wound contraction. The variables represented *herein* were tested in triplicate and replicated three times with similar findings each time (x420, before 30% reduction).

HA enhance the proliferation of fibroblasts from dermal tissue and promote the phenotypic changes associated with the migration of fibroblasts into a wound. The altered phenotype seen in Figure 2C is reminiscent of the change fibroblasts undergo when they invade a wound. This transformation to the myoblastic phenotype, characterized by an extensive microfilamentous network parallel to the long axis of the cell, is typical of wound matrix contraction. It is noteworthy that this response to HA occurred within a 24-hour period, as these cells had not been cultured with exogenous HA prior to mechanical disruption.

Cell motility is an essential element to many physiological processes, especially wound healing [19]. In vivo HA is a major constituent of the ECM produced by proliferating human dermal fibroblasts [4]. In our experiments, large amounts of exogenous HA may have rapidly established a fetal-like granulation tissue environment that contributed to the subsequent cell response. HA may foster cellular proliferation by binding mitogenic [20, 21] or chemoattractant factors present in serum and thereby act as an accessory receptor or cooperative ligand-binding pathway that increases the effective concentration of these factors at the cell surface. Additionally, HA may modulate transmembrane signaling [10] by binding directly to cell surface receptors, such as CD44 [22].

In our experiments, we noted a dramatic and reproducible change in the behavior of fibroblasts in the presence of HA. These observations are communicated via representative photomicrographs. Unfortunately, presumably due to the viscous matrix formed by these concentrations of HA, methods of cell enumeration routinely used

by our laboratory proved unsuitable in this experimental model. We are currently attempting to develop methods, based on cellular incorporation of vital dyes, to generate quantitative data that directly measure cell expansion. Further work will center on the investigation of growth rates and macromolecule synthesis by human dermal fibroblasts in HA matrices, in HA-collagen matrices, and in the presence of growth factor preparations from human platelets.

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