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Short Contribution

Hyaluronic acid modulates growth, morphology and cytoskeleton in embryonic chick skin fibroblasts

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ABSTRACT The action that hyaluronic acid (HA) exerts on cell proliferation was investigated in embryonic chick skin fibroblasts at different ages (7-14 days) and in different cell-cycle stages evaluated by flow cytometry (cells maintained with and without serum). Proliferation was estimated by ^{3}H-thymidine incorporation and cell counting. The results demonstrated hyaluronic acid inhibits cell multiplication in all different environmental conditions examined. The inhibitory effect of HA is more evident in 14-day than 7-day old fibroblasts. The ability of HA to modulate ^{3}H-thymidine incorporation did not involve a change in the time required for cells entering the S phase of the replicating cycle, but is due to a smaller number of cells entering in this phase. As the relationships between components of the extracellular matrix (ECM) and the cytoskeleton are known, parallel studies were carried out on some cytoskeleton proteins. Furthermore, by modifying the capacity of cells to adhere to the substrate, HA induced alterations in cell shape and in cytoskeleton components involved in these processes. We may hypothesize that HA, binding specific membrane receptors, affects cell adhesion and morphology inducing less receptivity of fibroblasts to mitogenic stimuli by transmembrane interactions with cytoskeleton.

KEY WORDS: *hyaluronic acid, proliferation, adhesion, cytoskeleton, fibroblasts*

Cell proliferation is influenced by the GAG composition of extracellular matrix (ECM,) particularly by HA (Moscatelli and Rubin 1975; Matuoka *et al.*, 1985; Yoneda *et al.*, 1988; West and Kumar 1989). We therefore administered HA to embryonic chick skin fibroblasts to ascertain whether altering the extracellular environment would induce modifications in cell growth in relationship to the cellular cycle stages, the initial seeding density and the specific incubation age. In addition, since changes in environmental conditions may cause variations in cell-substrate adhesion, cell morphology and cytoskeletal protein organization (Arena *et al.*, 1990), the alterations in cell shape and cytoskeleton pattern were studied in the same experimental model.

The results of the different experiments performed are given below.

Cell morphology and cell cycle analysis

Treatment of chick embryo fibroblasts with doses of HA between 50 and 250 µg/ml failed to induce changes in cell shape. Cells were elongated and adhered to the cell substrate as well as the control cells did. However, at a dose of 500 µg/ml, the cell bodies of about 30% of the population become rounded, due to the fact that the cells were less adherent to the substrate (data not shown). Cell cycle analysis of 11-day fibroblasts maintained in 199 medium without serum for 24 h revealed that 91% cells were in the G_0-G_1 phase, 7% in S and 2% in G_2-M , thus resulting synchronized. In fibroblasts cultured in 199 plus 10% serum, 72% were in the G_0-G_1 phase, 20% in S and 8% in G_2-M (Fig. 1).

Incorporation studies

Administration of 50-500 µg/ml HA to 7, 11 and 14-day fibroblasts synchronized in serum free 199 for 24 h provoked a significant drop in the percentage incorporation of ^{3}H-thymidine into DNA in 7 and 11-day fibroblasts only at the higher doses, whereas the drop was significant at all doses used in the 14-day fibroblasts (Fig. 2A). Cell number significantly diminished at all stages of incubation for all doses (data not shown).

Abbreviations used in this paper: ECM, extracellular matrix; GAG, glycosaminoglycans; HA, hyaluronic acid; CS, chondroitinsulfates; FCS, fetal calf serum; TCA, trichloroacetic acid; TRITC, tetramethylrhodamine isothiocyanate.

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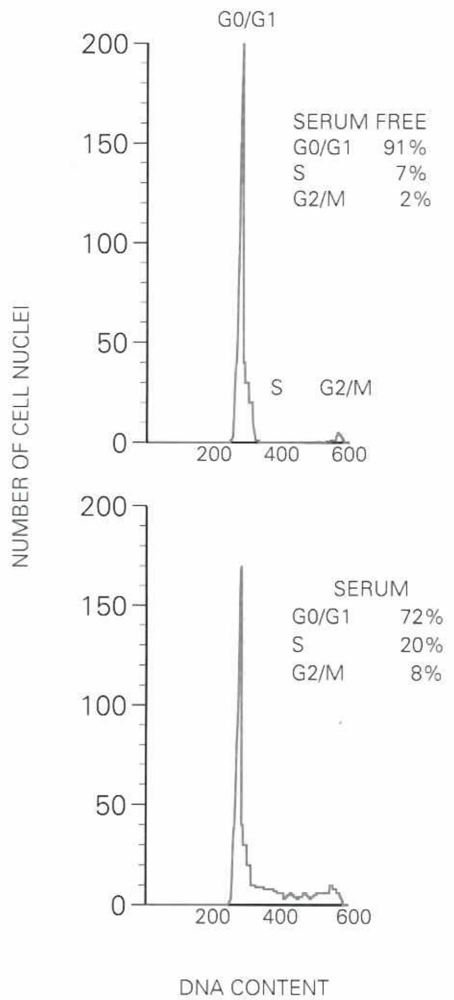

Fig. 1. Flow cytometric evaluation of cell-cycle of 11-day chick embryo fibroblasts.

Treatment of 11-day crowded fibroblasts cultured in 10% serum with 20, 50 and 250 μg/ml HA, determined a significant dose-dependent drop in the percentage of ^{3}H-thymidine incorporation, significant for 250 μg/ml at 48 h and for 50 μg/ml at 72 h, until a reduction about of 60% with 250 μg/ml HA is reached (Fig. 2B). 20 μg/ml HA did not modify the number of cells, but 50 or 250 μg/ml HA clearly reduce cell number until about 35% at 72 h at a higher dose (data not shown).

In sparse fibroblasts (Fig. 2C), HA caused a dose dependent drop in the percentage incorporation of ^{3}H-thymidine into the DNA at all the times under consideration. A reduction in cell number became evident at 48 h with the higher doses of HA, with a maximum at 72 h (data not shown).

To distinguish whether the inhibitory effect of HA was due to a slowing of the cell cycle or whether HA allowed fewer cells to enter the cell cycle, parallel cell cultures were synchronized in G_0-G_1 phase by treatment in serum free 199 for 24 h. Then 10% serum containing medium was added with or without HA (250 μg/ml) and 60 min pulses of ^{3}H-thymidine were administered at different culture times (6-12-18-24-30 and 36 h, Fig. 3A). The ^{3}H-thymidine uptake peak, which is the approximate time required to reach the S phase, was about 18 h in both control and HA treated cell. However, the number of cells was significantly lower in the HA treated cells at 18 h (Fig. 3B). Therefore, HA induced a lower number of cells entering the S phase rather than a slowing of the cell cycle, according to Goldberg and Toole (1987).

To confirm that the effect of HA was fairly specific, crowded fibroblasts were incubated for 48 h in 199+10% FCS with HA plus hyaluronidase or with chondroitin sulfates (CS) or DNA prior to the measurement of ^{3}H thymidine incorporation. The results showed a reversal of HA inhibition by Streptomyces hyaluronidase and no effects produced by CS or DNA (data not shown).

To exclude the possibility that HA could have exerted a cytotoxic effect in our cell system, ^{3}H-leucine incorporation studies were performed. They showed that 24 h treatment with 500 μg/ml HA did not provoke a significant reduction in neosynthesis of either endocellular proteins and those secreted into the medium (data not shown).

Cytoskeleton organization

Tubulin network (Fig. 4 A-B-C)

Tubulin staining revealed that the microtubules of control cells radiated outward from a perinuclear area to reach the cellular membrane and then ran parallel beneath it. 500 μg/ml HA provoked an evident rearrangement in the microtubular pattern only in the modified cells. Microtubules appeared more packed and thickened.

Actin network (Fig. 4 D-E-F)

Actin microfilaments of control cultures and of those added with 50 and 250 μg/ml HA, formed delicate bundles that extended beneath the membrane and traversed the cell in various directions (stress fibres). Treatment with 500 μg/ml HA provoked a loss of stress fibres in the modified cells. Microfilaments clearly appeared in the cell boundary and along filopodia or converted to cytoplasmic aggregates.

α–Actinin network (Fig. 4 G-H)

α-actinin was localized in a punctate manner in the cytoplasm (following the disposition of actin microfilament bundles) and in the cellular membrane of control cells. The microfilament bundle-associated α-actinin was lost in 500 μg/ml HA treated cells, while membrane-associated α-actinin was detectable at the edge of the cell and diffused perinuclearly.

These results confirm that ECM components are able to modulate growth, by affecting cellular shape (Fisher and Solursh 1979; Hormann and Jelinic, 1981; Abatangelo *et al.*, 1982; Bissel *et al.*, 1982; Hamaty *et al.*, 1989). It is possible that relative concentration fluxes of HA in the extracellular matrix during development represent a potential factor which is able to interfere with mechanisms of cell-substrate interaction and with cellular response to mitogenic factors.

Experimental Procedures

Cell culture

Primary cultures of fibroblasts obtained from the dorsum of chick embryos were prepared as previously described (Bodo *et al.*, 1988) Briefly, fibroblasts from chick embryos at the 7th, 11th and 14th incubation days were

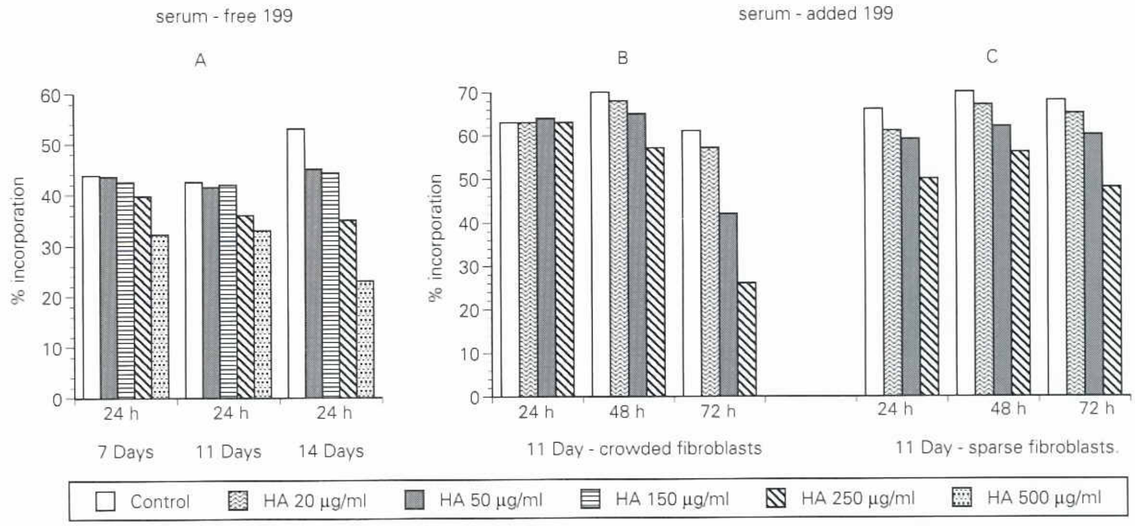

Fig. 2. Percent incorporation (d.p.m. TCA insoluble fraction/total radioactivity x 100) in HA treated chick embryo fibroblasts.

seeded at concentration of 9×10^5 cells/ml in 199 medium containing 10% fetal calf serum (FCS), maintained in a 5% CO_2 humidity saturated atmosphere for 24 h (plating) and then for a further 24 h without serum in medium 199 alone or medium 199 plus high molecular weight HA (purified from rooster comb, Sigma Chemical Company, St. Louis, USA) in scaled up concentrations from 50 to 500 μg/ml. Parallel cultures of 11 day-old fibroblasts, seeded at a concentration of 3×10^5 or 9×10^5 cells/ml to obtain sparse or crowded cell cultures, were maintained in medium 199 containing 10% FCS for 24, 48 or 72 h with and without HA (20, 50 or 250 μg/ml). Cell number was estimated by crystal violet at the end of culture time, according to Gillies *et al.* (1986).

Cell cycle analysis

Analysis of the cell cycle was evaluated in 11-day fibroblasts maintained for 24 h after plating, for 24 h in two different environmental conditions: in 199 without serum or in 199+10% FCS. Cells were washed twice with cold PBS and 2 ml fluorochrome solution (propidium iodide 0.05 mg/ml dissolved in 0.1% Na citrate with 0.1% Triton X-100 added) (Fried *et al.*, 1978).

^{3}H-thymidine incorporation

Fibroblasts maintained with or without serum were incubated in the presence and absence of HA (from 20 to 500 μg/ml) and 1 μCi/ml ^{3}H-thymidine (Radiochemical Amersham, 13.4 Ci/mmol specific activity) or ^{3}H-leucine (Radiochemical Amersham, 197 Ci/mmol specific activity) was added during the last 3 h of culture. The medium was discarded and cell monolayers washed and solubilized in 0.5 M NaOH. One aliquot was precipitated with 10% trichloroacetic acid (TCA) for 30 min at 4°C, filtered on glass fiber filters (Millipore 0.45 μm) and washed with cold 5% TCA (TCA insoluble fraction). The relationship between ^{3}H-thymidine incorporation into TCA insoluble fraction and total radioactivity was calculated as percent incorporation. Results of ^{3}H-leucine incorporation were calculated as dpm/mg proteins calculated according to Lowry, 1951. In parallel sets, HA was digested with Streptomyces hyaluronidase (100 U/ml) (Miles Laboratories, Elkhart I.N.) at 37° for 24 h and then heated in bath of boiling water for 10 min before addition to the cultures. Further comparisons were made using chondroitin sulfate (250 μg/ml - mixed isomers of chondroitin-4-sulfate and chondroitin-6-sulfate, Sigma) or highly polymerized DNA (0.01 mg/ml-from calf thymus, BDH Chemicals Ltd., Poole, England).

Immunofluorescence procedure

Control and HA (50, 250 and 500 μg/ml) treated cells were processed according to Arena *et al.* (1990). Normal swine serum was obtained from ICN-Immunobiologicals, Lisle, Israel, rhodamine-labeled phalloidin (Sigma Chemical Co., St. Louis, USA) was used to visualize actin; monoclonal anti-(α-ß) tubulin, anti-α-actinin and anti-fibronectin antibodies were purchased from Bio-Makor (Rehovot, Israel) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated affinity purified anti-mouse antibody from Cappel (Cochranville, USA).

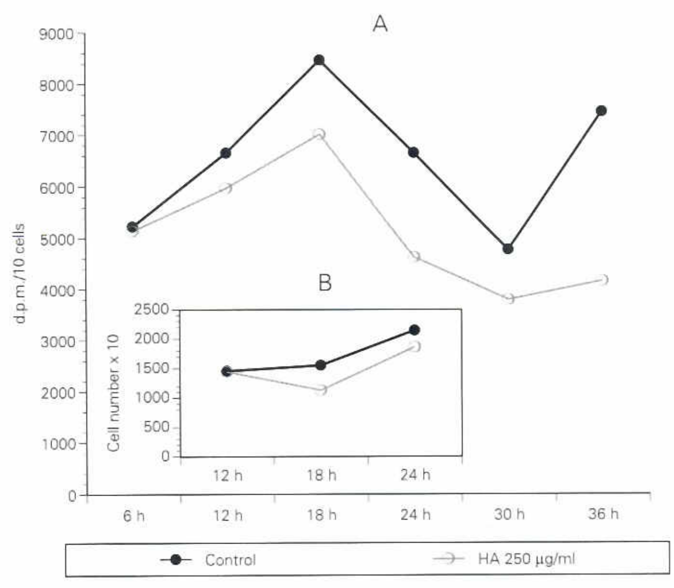

Fig. 3. Effect of HA on ^{3}H-thymidine incorporation (A) and cell number (B) of 11 day fibroblasts synchronized by treatment with serum-free 199. *After 24 h, 199+10% FCS was added and, at each indicated period, ^{3}H-thymidine was added for 60 min. Values are expressed as d.p.m. of ^{3}H-thymidine/number of cells.*

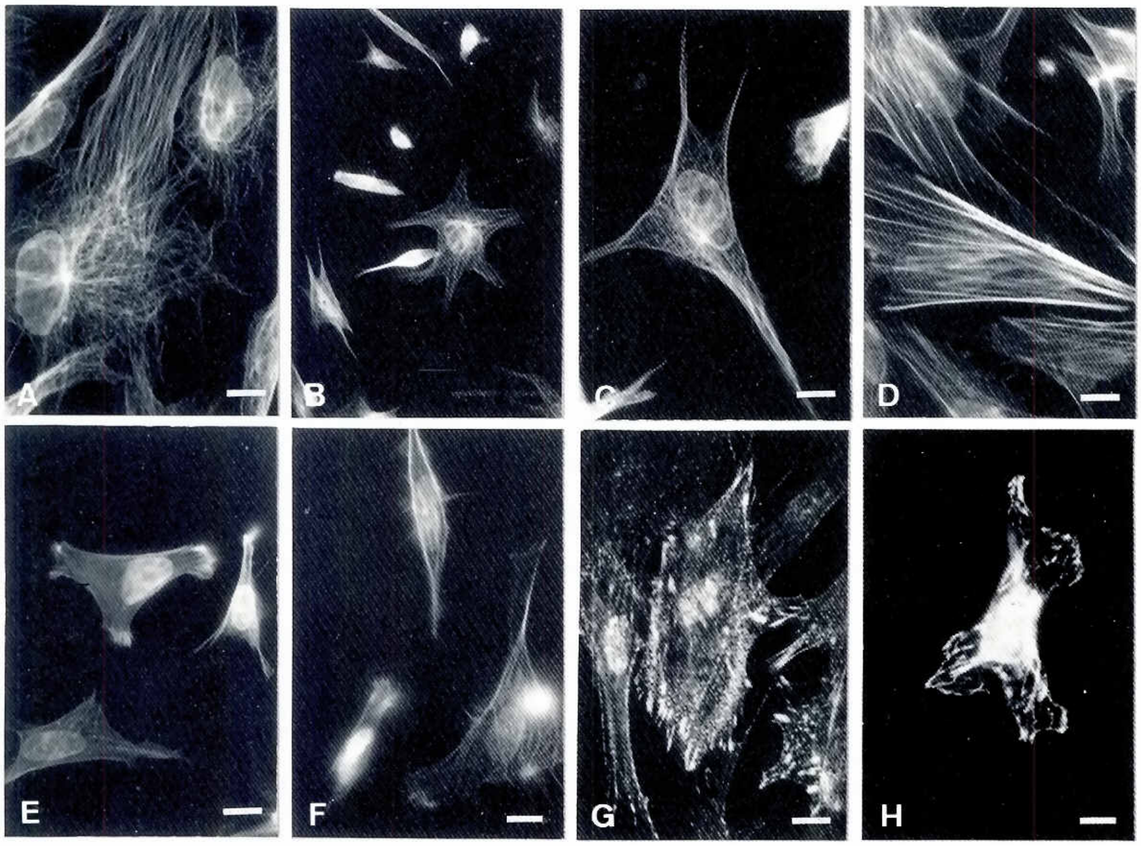

Fig. 4. Immunofluorescence staining of (A, B, C) tubulin, (D, E, F) actin and (G, H) α-actinin pattern in chick embryo fibroblasts maintained in 199+10% FCS with and without 500 μg/ml HA for 24 h. (A, D, G) *Control;* **(B, C, E, F, H)** *HA treated cells. Bar, 10 μm.*

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