Changes in the expression of *c-myc*, *RB* and tyrosine-phosphorylated proteins during proliferation of NIH 3T3 cells induced by hyaluronic acid

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Abbreviations: IL-1, interleukin-1; TNF- α , tumor necrosis factor- α ; FAK, focal adhesion kinase; ATCC, American type culture collection; DMEM, Dulbesco's modified Eagle's medium; SRB, sulforhodamin B; pRb, RB product

Abstract

We have shown that hyaluronic acid stimulates the proliferation of quiescent NIH 3T3 cells. We have shown that treatment of 1 mg/ml hyaluronic acid results in increase of tyrosine phosphorylation of two proteins, MW 124 kDa and 60 kDa as detected by anti-tyrosine antibodies by Western blot analysis. Maximum phosphorylation occurred within 2 h after addition of 1 mg/ml hyaluronic acid. Stimulation of proliferation was also accompanied by increase in *c-Myc* protein, which was inhibited by amiloride, an inhibitor of Na⁺/H⁺ antiporter and EGTA and increase in the steady state level of pRb, the RB gene product. These results suggest that the intracellular signal transduction pathways that mediate the stimulatory effects of hyaluronic acid on cellular proliferation are similar to those of growth factors.

Keywords: proliferation, tyrosine phosphorylation, *c-myc*, pRb, hyaluronic acid

Introduction

Hyaluronic acid, an extracellular matrix glycosaminoglycan, is an linear polysaccharide containing glucuronic acid and N-acetylglucosamine as a repeating unit. Hyaluronic acid provides stability and elasticity to the extracellular matrix by interaction with proteoglycans and other matrix components (Knudson and Knudson, 1993). Hyaluronic

acid has been thought to be important not only as a structural role in extracellular matrix, but also as biological roles. The main biological function of hyaluronic acid is still unknown but recently several functions have been elucidated. Brecht *et al.* (1986) have suggested that hyaluronic acid is involved in the mitotic process. Hyaluronic acid also has been shown to promote cell motility *in vitro* and contribute to the motile responses of cells during wound repair, tumor invasion and tissue morphogenesis (Turley, 1984; Weigel *et al.*, 1986; Toole *et al.*, 1989). Hyaluronic acid has been previously been linked with both transformation and metastatic process (Turley, 1992). Hyaluronic acid has also been recognized in clinical medicine. Analysis of serum hyaluronic acid is promising in the diagnosis of liver disease and various inflammatory conditions. Interstitial edema caused by accumulation of hyaluronic acid may cause dysfunction in various organs (Laurent and Fraser, 1992). Also hyaluronic acid has been reported to regulate the expression of several cytokine genes. In bone marrow derived macrophages, soluble hyaluronic acid stimulated IL-1, TNF- α , and insulin like growth factor-1 mRNA transcript expression (Hiro *et al.*, 1986; Noble *et al.*, 1993).

It has been proposed that hyaluronic acid mediates its biological function by interacting with hyaluronic acid binding proteins in the extracellular matrix and hyaluronic acid receptors on the cell surface (Aruffo *et al.*, 1990; Harwick *et al.*, 1992). Interactions between hyaluronic acid and the cell surface receptors generate a variety of biochemical signaling events including transient increase of tyrosine phosphorylation of several proteins leading to focal adhesion turnover (Hall *et al.*, 1994) and selective expression of genes (Noble *et al.*, 1993). Focal adhesions are sites of extracellular matrix receptor-cytoskeletal interactions, where second messenger signaling commonly occurs in response to extracellular matrix and some growth factors. These structures are believed to be critical for the regulation of growth and cell motility (Burrige *et al.*, 1992). Focal adhesion kinase (FAK), pp125^{FAK} a tyrosine kinase, recently localized in focal adhesions, has been implicated in several signaling pathways that involve extracellular matrix receptors as well as in transformation (Schaller *et al.*, 1992; Schlaepfer *et al.*, 1994). Relationship of FAK and ras signaling pathway is well studied in fibronectin and integrin interaction on NIH 3T3 cells. Fibronectin binding to integrins on NIH 3T3 cells promotes *c-src* and FAK association and increase FAK tyrosine phosphorylation (Guan and Shalloway, 1992; Schaller *et al.*, 1992) and FAK protein tyrosine kinase activity (Guan and Shalloway, 1992). Phosphorylation of FAK

upon fibronectin stimulation creates an SH2-binding site for GRB2 which may link integrin engagement to the activation of Ras signal transduction pathway (Schlaepfer *et al.*, 1994).

These results indicate that hyaluronic acid may regulate the cell proliferation through activation of FAK-Ras pathway. However, cell proliferation affected by hyaluronic acid is not established and mechanism by which this occurs are not fully understood. In this present study, we have attempted to elucidate the intracellular signal transduction pathways that mediate the growth stimulatory effects of hyaluronic acid. We have focused on the changes in the expression of *c-myc* and RB and tyrosine phosphorylation

Materials and Methods

Cell culture

NIH 3T3 cells were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modification of Eagle's minimal essential medium (DMEM) supplemented with 5% bovine calf serum (Gibco, Gaithersburg, USA), 50 µg/ml penicillin, and 50 unit/ml streptomycin in a humidified incubator containing of 5% CO₂ at 37°C.

Cell proliferation

Quiescent cells were obtained by serum deprivation (0.5% BCS) for two days. Cell proliferation was determined by using sulforhodamin B (SRB, Sigma, St. Louis, USA) assay. SRB assay was performed as described (Rubinstein *et al.*, 1990). After quiescent NIH 3T3 cells were treated with hyaluronic acid (prepared from culture of *Streptomyces equi*: ATCC 9528 as described in Yoon *et al.*, 1993) for one day, media was aspirated and 10% trichloroacetic acid was added. After 1 h incubation at 4°C, plate was washed 5 times by using tap water and air dried. Cells were stained with 0.4% (w/v) SRB for 30 min at room temperature and washed 5 times by using 1% acetic acid. Bound SRB was solubilized with 10 mM Tris and absorbance was measured at 520 nm.

Western blot analysis

After quiescent cells were treated with hyaluronic acid or hyaluronic acid plus amiloride (Sigma, St. Louis USA) or EGTA. NIH 3T3 cells were lysed directly in SDS loading buffer, heated to 100°C for 5 min and protein was quantitated by using Bio-Rad microassay kit (Bio-Rad, Hercules, USA). Same amount of protein was resolved by SDS-PAGE. After electrophoresis, the separated polypeptides were electrophoretically transferred to nitrocellulose membrane (Schleicher & Schull, Keene, USA) by using electrotransfer kit (Hoefer, San Francisco, USA). The membrane was immersed in blocking buffer [0.2% (w/v) I-block™, 1XPBS, 0.1% (v/v) Tween-20] for 1 h at room

temperature to block nonspecific binding and incubated for 1 h with each primary antibody. The antibodies were rabbit polyclonal pRb antibody (Ab-2, oncogene science, New York, USA), rabbit polyclonal anti-c-Myc antibody (UBI, New York, USA), and mouse monoclonal anti-phosphotyrosine antibody (Transduction Laboratories, Lexington, USA). The membranes were washed in 0.1% (v/v) Tween-20/PBS and incubated with biotinylated secondary antibody in blocking buffer for 1 h at room temperature. After washing, membranes were incubated in alkaline phosphatase conjugated streptavidin containing blocking buffer for 30 min at room temperature. Bands were visualized by incubating the membrane in substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Results and Discussion

The effect of hyaluronic acid on the proliferation of NIH 3T3 cells

Recently, it has been known that hyaluronic acid and other glycosaminoglycans have effects on cell proliferation and transformation (Turley, 1992; Knudson and Knudson, 1993). Overexpression of one of isoforms of hyaluronic acid receptor CD44 increase primary and secondary tumor growth and also cells overexpressing RHAMM resemble *ras*-transformed fibroblasts (Hall *et al.*, 1995). However, the molecular mechanisms underlying hyaluronic acid regulation of cell proliferation are unknown. For elucidation of the signal transduction pathway that is responsible for this process, first, we observed the effect of hyaluronic acid on NIH 3T3 cell proliferation. We treated various concentration of hyaluronic acid to quiescent NIH 3T3

Figure 1. Effect of hyaluronic acid on the proliferation of NIH 3T3 cells. After quiescent NIH 3T3 cells were incubated with 0.5-1.5 mg/ml hyaluronic acid for 24 h. SRB assay was performed. This is a representative plot of five similar experiments. Results are expressed as the percentage of control cells which are cultured in 0.5% serum media.

cells for 24 h and SRB assay was performed. Hyaluronic acid stimulated proliferation of NIH 3T3 cells 2.0-fold and maximum effect was observed at 1 mg/ml hyaluronic acid (Figure 1).

The effect of hyaluronic acid on the tyrosine phosphorylation

One of the best known responses stimulated by hyaluronic acid is tyrosine phosphorylation of several proteins (Hall *et al.*, 1994; Hall *et al.*, 1995). To analyze the signaling through tyrosine phosphorylation in hyaluronic acid treated quiescent NIH 3T3 cells, we have studied phosphotyrosine containing proteins with anti-phosphotyrosine antibody (Figure 2). Hyaluronic acid stimulated the phosphorylation of 124 kDa and 60 kDa proteins. Molecular weights of these proteins were similar to those of FAK (125 kDa) and c-src (60 kDa) (Turley, 1989; Burridge *et al.*, 1992). Tyrosine phosphorylation of these proteins have been implicated to be related in hyaluronic acid induced cell motility (Hall *et al.*, 1994, 1995). Phosphorylation increases within one hour after addition of 1 mg/ml hyaluronic acid and maximum effect was observed after two hours. In contrast to our result, Hall *et al.* (1994) reported that phosphorylation peaked within one minute of hyaluronic acid stimulation. They used much lower concentration (10 ng/ml) of hyaluronic acid than that we used. We also observed minor stimulation of phosphorylation within three minutes (data not shown). This different time dependent phosphorylation may be resulted from stimulation of different receptor according to different concentration of hyaluronic acid. Further investigation should be performed to identify these two proteins and to confirm that phosphorylation of these proteins are critical for proliferation of NIH 3T3 cells by using the inhibitor of

tyrosine kinases.

The effect of hyaluronic acid on c-Myc protein expression

The expression of *c-myc* is closely associated with growth in various cell types. The expression of *c-myc* is induced transiently with maximal expression occurring two hours after serum stimulation at the level of mRNA (Moore *et al.* 1986). To investigate whether hyaluronic acid stimulates this important proto-oncogene as other mitogens, we treated quiescent NIH 3T3 cells with 1 mg/ml hyaluronic acid and Western blot analysis was performed. Figure 3 shows that hyaluronic acid causes increase of steady state level of c-Myc protein within one hour. Rapid increase of c-Myc protein by hyaluronic acid suggests that this is important signal of hyaluronic acid necessary for cells to proceed from G₀ to G₁.

The effect of amiloride, an inhibitor of Na⁺/H⁺ antiporter and EGTA on the hyaluronic acid induced c-Myc protein expression

To determine whether the intracellular signal transduction pathways, stimulated by hyaluronic acid are similar to those of growth factors, we investigated the significance of two important signaling molecules, Na⁺/H⁺ and Ca²⁺ in hyaluronic acid induced c-Myc protein expression by using amiloride, an inhibitor of Na⁺/H⁺ antiporter, and EGTA. One of the important early responses of cultured fibroblasts to growth factors is the activation of Na⁺/H⁺ antiporter which leads to a rise in cytoplasmic pH. By using amiloride, the importance of Na⁺/H⁺ antiporter in the induction of *c-myc* and also cell proliferation was found (Moon and Kim, 1993). Treatment of amiloride and EGTA with hyaluronic acid inhibited the increase of c-Myc protein induced by hyaluronic acid (Figure 4). We also found that amiloride and EGTA inhibited NIH 3T3 cell proliferation induced by hyaluronic acid (data not shown). This result supports that hyaluronic acid and growth factors stimulated the common signaling pathway to induce cell proliferation.

Figure 2. Effect of hyaluronic acid on tyrosine phosphorylation. Quiescent NIH 3T3 cells were incubated with 1 mg/ml hyaluronic acid for 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), or 3 h (lane 4) before cell lysis. 5 µg of total protein was electrophoresed on SDS-PAGE gel and transferred to nitrocellulose membrane and detected by using anti-phosphotyrosine antibody (A). The equivalent gel was stained with Coomassie blue (B).

Figure 3. Effect of hyaluronic acid on the steady state level of c-Myc protein. Quiescent NIH 3T3 cells were incubated with 1 mg/ml hyaluronic acid for 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), or 3 h (lane 4) before cell lysis. Western blot analysis was performed with anti-c-Myc antibody as described in Material and Methods. The arrow indicates the 64 kDa c-Myc protein.

Figure 4. Effect of amiloride and EGTA on the hyaluronic acid induced c-Myc protein. Quiescent NIH 3T3 cells were incubated with 1 mg/ml hyaluronic acid in the presence of 500 μ M amiloride (1-4) or 1 mM EGTA (5-8) for 0 h (lane 1 and 5), 1 h (lane 2 and 6), 2 h (lane 3 and 7), or 3 h (lane 4 and 8) before cell lysis. Western blot analysis was performed with anti-c-Myc antibody as described in Material and Methods. The arrow indicates the 64 kDa c-Myc protein.

Figure 5. Effect of hyaluronic acid on the steady state level of pRb. Quiescent NIH 3T3 cells were incubated with 1 mg/ml hyaluronic acid for 0 h (lane 1), 6 h (lane 2), 12 h (lane 3), or 24 h (lane 4) before cell lysis. Western blot analysis was performed with anti-pRb antibody as described in Material and Methods. The arrow indicates the 105 kDa pRb.

The effect of hyaluronic acid on RB protein expression

In addition to proto-oncogenes, the other important regulators of cell cycle are anti-oncogenes. RB is one of the well studied anti-oncogenes. pRb acts as a growth suppressor and prevent G1-S transition. The activity of pRb has been known to be regulated primarily by cell cycle dependent phosphorylation and only the hypophosphorylated form of pRb is able to interact with cellular proteins including E2F (DeCaprio *et al.*, 1989). However, recently it has been shown that the expression of RB gene is subjected to cell cycle regulation and this process occurred through E2F binding site, located in RB promoter (Shan *et al.*, 1994). To elucidate the relationship between hyaluronic acid and the pRb, we investigated the effect of hyaluronic acid on the steady state level of pRb by Western blot analysis. Figure 5 shows that hyaluronic acid increases the amount of pRb. This result supports the previous evidences which showed that RB expression increases during cell cycle progression. It has been previously shown that G₀-differentiated cells have less pRb than proliferating, immature cells (Yen *et al.*, 1994) and G₁ cells have less pRb than cell in other cell cycle phase (Yen and Chandler, 1992). Our result provides further evidence that the expression of the RB gene is subjected to cell cycle regulation. It will be interesting to

study the mechanism by which hyaluronic acid increase the expression of RB.

Rapid responses of hyaluronic acid, such as stimulation of tyrosine phosphorylation and increase of c-Myc protein level which was dependent on Na⁺/H⁺ and Ca²⁺, as shown in this paper, suggest that hyaluronic acid stimulates the cellular proliferation by stimulation of signal transduction pathway through interaction with specific receptor.

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