Effect of Genistein on ATP-induced Cell Retraction and Rounding in Human Chang Liver Cells

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Summary: Extracellular ATP has been previously reported to induce cell retraction and stimulate DNA synthesis in human Chang liver cells via phosphoinositide (PI) signal transduction. The ATP-treated cell assumes a rounded morphology and is tethered to the substratum by microvillous anchors as demonstrated in this study by scanning electron and confocal microscopy. The addition of 0.5 mM genistein, which is known to completely abolish extracellular ATP-stimulated DNA synthesis in Chang liver cells, did not inhibit ATP-induced cell rounding. The results suggest that the seemingly conjoint responses of ATP-stimulated DNA synthesis and cell rounding could be dissociated.

Genistein, a known tyrosine kinase inhibitor (Akiyama *et al.*, 1987) has been reported to inhibit DNA synthesis in phosphotyrosine kinase (PTK) dependent and non-PTK dependent pathways (Gaudette and Holub, 1990; Sit *et al.*, 1992a). Because of its antiproliferative activity, this isoflavone has been cited as a potential anti-cancer agent in the chemoprevention and chemotherapy of human breast and prostate cancers (Barnes *et al.*, 1995) and experimental breast cancer (Lamartinere *et al.*, 1995).

Genistein at 0.5 mM concentration has been shown to completely abolish ATP-stimulated DNA synthesis but not intracellular alkalinization in Chang liver cells (Sit *et al.*, 1991). It is well established that extracellular ATP binds P2-purinergic receptors (Burnstock, 1990) and activates phosphoinositide-specific phospholipase C (PLC) (Dubyak *et al.*, 1988) which in turn generates the PI second messengers, diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP₃). DAG is known to stimulate Ca²⁺/phospholipid dependent protein kinase C (PKC) which causes allosteric activation of the Na⁺/H⁺ antiporter (Ober and Pardee, 1987; Nishizuka, 1989; Bay and Sit, 1993; Bay *et al.*, 1996a).

Since upshifting of intracellular pH (pH_i) induced by extracellular ATP has been associated with cell retraction and rounding (a process which is important in the metastatic cascade) (Sit *et al.*,

1992b; Bay *et al.*, 1996b), the effect of genistein on ATP-induced cell rounding was evaluated.

Materials and Methods

Cell cultures

Human Chang liver cells (American Type Culture Collection, ATCC CCL 13) were cultured in Dulbecco's modified Eagles medium (DMEM, Sigma, USA) supplemented with 10% foetal bovine serum (Cytosystems, Australia) and 10% CO₂ in air. Cells were seeded at 3 × 10⁵ cells per 25 cm² culture flask (Costar, USA) by the Filamatic dispenser (National Instrument, USA).

ATP induced cell rounding with and without genistein

Parallel 25 cm² cultures were rinsed with 2 × 10 ml Na⁺-HEPES buffer [140 mM NaCl, 10 mM HEPES (Sigma) adjusted to pH 7.4 with NaOH] and overlaid with 2 ml Na⁺-HEPES buffer containing 7 mM ATP (adenosine 5'-triphosphate, sodium salt, Sigma) with and without 0.5 mM genistein (Funakoshi Pharmaceutical, Japan). Cultures were incubated at 37°C for the required duration in time response. Cells were fixed after 0, 4, 8, 15 min in NAMETCA (140 mM NaCl in 70% methanol acidified with 3% trichloroacetic acid) and stained with Giemsa's stain [Gurr's improved R66 (BDH, UK)]. Slides were examined in a Nikon microscope

on line with the Quantimet image analysis system. Cell profile area, perimeter and cell rounding were measured using the QUIPS programme (Bay *et al.*, 1996b).

Scanning electron microscopy

Cells were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 30 minutes. This was followed by osmication with 2% osmium tetroxide as previously described (Bay *et al.*, 1997). After dehydration, cells were dried in the Balzers Critical Point Dryer model CPD030, using liquefied carbon dioxide. Samples were coated with 20 nm of gold in a Balzers Sputter Coater before examination in a Philips SEM 515 scanning electron microscope.

Confocal microscopy

Imaging was done in the BioRad MRC600 confocal system equipped with a 25 mW argon laser. Cells were visualized in a Bio-Rad M17 upright microscope. The light output was reduced to 3% of total power by a neutral density filter.

Results and Discussion

Added ATP caused a reduction in the cell profile area and perimeter in the time response (Fig. 1). The cell profile area reduced by approximately 75% from a mean of 1045 μm^2 to 141 μm^2 at the end of 15 minutes (Fig. 1A). The cell profile perimeter diminished by approximately 70% from a mean of 320 μm to 47 μm at the end of 15 minutes (Fig. 1B). The addition of 0.5 mM genistein to the ATP incubation buffer had no significant effect on the reduction in profile area and perimeter ($p < 0.05$, ANOVA).

Concurrent with the reduction in profile area and perimeter, ATP-treated Chang liver cells undergo a flat to round change in morphology as evidenced by the increasingly rounded cell profile (Fig. 2). No significant difference was observed in the cell rounding response of ATP-treated cells in the presence or absence of genistein. At the end of 15 minutes, ATP-treated cells were retracted from the substratum and characterized by multiple long thin processes which maintained contact with the substratum (Fig. 3). The progression of untreated human Chang liver cells from a flattened anchored state into a rounded morphology with ATP incubation is shown in Fig. 4. Cells round up as a result of a drastic reduction of cell surface area due to massive internalization of plasma membrane by antiport mediated endocytosis (Sit *et al.*, 1994; Bay *et al.*, 1996b).

ATP is also known to stimulate DNA synthesis

via activation of PKC, a serine-threonine kinase (Nishizuka, 1992; Azzi *et al.*, 1992). It appears that genistein is able to completely suppress ATP-stimulated DNA synthesis (Sit *et al.*, 1991) by its inhibitory action on tyrosine kinase (Akiyama *et al.*, 1987). Genistein has been shown to directly reduce the phosphorylation of oncogenic proteins and other substrates (Nishimura, 1995).

However, genistein did not inhibit ATP-induced cell retraction and rounding. Release of cells from anchorage to the substratum must occur before cells can disseminate to distant foci (Juliano, 1987). The observation that the seemingly conjoint responses of ATP-induced cell rounding and stimulated DNA synthesis are separable, suggest that DNA damage or suppression in cancers by chemotherapeutic agents (Berger & Hirschler, 1992) may not necessarily affect their metastatic potentials.

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Explanation of Figures

Plate I

Fig. 1. Profile area and perimeter of human Chang liver cells in 7 mM ATP-treated cells with and without 0.5 mM genistein: time response. (A) Cell profile area. (B) Cell profile perimeter. Error bar = 2SE.

Fig. 2. Roundness form factor according to the formula $(\text{perimeter})^2/4\pi(\text{area})$. Roundness form factor in untreated Chang liver cells was 6.7 and in ATP treated cells were 1.2 (without genistein) and 1.3 (with genistein). When the value is 1, the cell is perfectly round. Error bar = 2SE.

Plate II

Fig. 3. Scanning electron micrograph of Chang liver cells. (A) Untreated Chang cell. (B) ATP-treated Chang cell after 15 minutes. Bar = 10 μ m.

Fig. 4. Confocal microscopic images of ATP-induced cell rounding: time response. (A) 0 minutes (B) 2 minutes (C) 4 minutes (D) 8 minutes (E) 15 minutes. Bar = 10 μ m.