Role of Tyrosine Kinase and Phosphotyrosine Phosphatase in Growth of the Intestinal Crypt Cell (IEC-6) Line (43555)

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Abstract. **The roles of gastrin and sodium vanadate in proliferation were examined in cultured IEC-6 cells that are mitotically active and derived originally from jejunal crypts of the rat intestine. Incubation of the cells in the presence of gastrin at a concentration of 250 ng/ml or of sodium vanadate at a concentration of 0.2 m*M* leads to a 60% increase in cell growth in 24 hr. The stimulated growth in both cases was inhibited by genistein, a tyrosine kinase inhibitor. Incubation in the presence of gastrin and sodium vanadate together produced a small, albeit significant, potentiation of growth of the cells. Gastrin as well as sodium vanadate also promoted the phosphorylation on tyrosine of a similar group of proteins with molecular masses of 42, 45, 52, 60, 78, and 120 kDa. The phosphorylations were rapidly occurring as early as 5 min and lasted for only 15 min. Several proteins were detected in normal IEC-6 cells, including GTPase activating protein, raf1 kinase, phospholipase C γ-1, and phosphoinositide 3-kinase. The results suggest that gastrin and sodium vanadate induce growth of IEC-6 cells by stimulation of tyrosine kinase and/or inhibition of tyrosine phosphatase. The gastrin and sodium vanadate effects also involve the phosphorylation of a number of proteins, the identities of which are not known at present but may include some of the kinases that are frequently associated with cell growth, such as mitogen-activated protein kinase, raf1 kinase, phosphoinositide 3-kinase, and others.** [P.S.E.B.M. 1993, Vol 202]

The intestinal epithelium is a mitotically active tissue that can be stimulated by hormones, growth factors, and luminal nutrients (1). The crypt cells are believed to be the progenitor cells of the epithelium, capable of rapid proliferation, differentiation, and migration in response to a variety of regulatory factors (1–4). The mechanisms by which such factors affect growth are, however, unclear. To elucidate the molecular mechanism of growth in crypt cells, we tested the effect of gastrin on the protein tyrosine phosphorylation and cell proliferation in cultured IEC-6 cells and the relationship of this activity to the action of sodium vanadate, which is a known protein phosphotyrosine phosphatase inhibitor (5). The use of a cultured cell line (6) for the present study was necessary since the isolation of a viable and homogeneous population of intestinal epithelial cells has been described to be quite difficult (7). We used the IEC-6 cell line because it is, as described by Quaroni *et al.* (6), a nontumor continuous cell line that retains many of the antigenic and physical properties of intact crypt cells of the intestinal epithelium (6). The IEC-6 cells also respond to gastrin and epidermal growth factor stimulation by an increase in cell replication and DNA synthesis (8). The present studies were undertaken to investigate the role of tyrosine kinase and/or phosphotyrosine phosphatases in the gastrin-induced effect on proliferation of IEC-6 cells. The rationale behind these studies are many and relate, in part, to a recent study of cultured colonic mucosal tissues by Majumdar (9), who found that the induction of tyrosine kinases and the tyrosine phosphorylation of certain proteins may be necessary for mediating the trophic effect of gastrin. Also Narayan and Singh (10) as well as us (11), in abstract forms, have demonstrated that gastrin stimu-

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lates tyrosine kinase activity and tyrosine phosphorylation of a number of proteins in IEC-6 cells. In this communication, we present evidence to suggest that gastrin and sodium vanadate respectively stimulate growth of IEC-6 cells by activation of tyrosine kinase and inhibition of phosphotyrosine phosphatase.

Materials and Methods

Preparation of Cells. The rat intestinal cell line IEC-6 was obtained from the American Type Culture Collection (Rockville, MD). The cells were in their sixth to seventh number of passages and were cultured in a water-saturated atmosphere with 10% CO_2 at 37°C. The cells were cultured in 75-cm polystyrene flasks containing Dulbecco's modified Eagle's medium [DMEM] supplemented with 4 m*M* glutamine, 10 units/ml of insulin, 10% dialyzed fetal bovine serum, 50 units/ml of penicillin, 50 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B [all from Gibco, Grand Island, NY]. The cells were harvested by trypsinization and reseeded in wells at a density of 0.5–1.0 million cells/well. Duplicate samples of the reseeded cell suspension were obtained and counted using a hemocytometer. These initial cell counts represented the control reference counts prior to incubation under various conditions. The cells were then allowed to reach 50% confluence and incubated further in triplicate wells in the presence or absence of gastrin (G-17; Peninsula Laboratory, Belmont, CA); genistein (Upstate Biotechnology, Inc., Lake Placid, NY), or sodium vanadate (sodium orthovanadate; ICN Biochemicals, Inc., Plainview, NY). Proliferation studies were conducted, at appropriate time intervals, on cells that were detached with 0.25% trypsin-EDTA (Gibco), then incubated in 0.5 ml of the enzyme for a period of 10 min. The cells were then suspended in DMEM and counted in duplicates. The data are expressed as mean ± SE of the percentage of change in the experimental over the control counts. Cell viability was determined by the trypan blue exclusion method after a 1-min exposure of the cells to a DMEM solution containing 0.04% trypan blue.

Protein Tyrosine Phosphorylation. Protein tyrosine phosphorylation was measured as described previously (12). Briefly, cells were grown as described above. They were exposed to gastrin at 250 ng/ml for 5, 10, and 15 min and the reaction was terminated by placing the plates on ice and the media were removed. After rinsing twice with ice-cold Hanks' balanced salt solution (JRH Biosciences, Lenexa, KS), the cells were lysed by rocking at 4°C for 20 min in 1 ml of ice-cold lysis buffer (1% Triton X-100, 20 m*M* Tris (pH 8.0), 13 m*M* sodium pyrophosphate, 1 m*M* phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin, 2 m*M* sodium vanadate, and 10 μg/ml of aprotinin). The lysate was removed and spun in a microfuge for 10 min at 4°C. The supernatant was tested for its protein content using a Bio-Rad DC protein assay (Bio-Rad, Richmond, CA). Samples of supernatant were then prepared using 40 μg of protein in sample buffer (8% sodium dodecyl sulfate, 10% glycerol, 86 m*M* Tris [pH 6.8], 0.5% bromphenol blue, and 10% 2-mercaptoethanol). The samples were boiled for 5 min. Proteins were resolved by electrophoresis on 8% sodium dodecyl sulfate-polyacrylamide gels, transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA), and probed with affinity-purified mouse monoclonal antiphosphotyrosine antibody (Upstate Biotechnology), followed by chemiluminescence detection (electrogenerated chemiluminescence; Amersham, Arlington Heights, IL) using anti-mouse horseradish peroxidase-conjugated antibody (Amersham).

Identification of Proteins. Individual proteins were identified by sequential probing of stripped immunoblots with anti-human GTPase activating protein, anti-human raf1 kinase, anti-rat phosphoinositide 3-kinase, and anti-bovine phospholipase C γ-1 (Upstate Biotechnology) followed by electrogenerated chemiluminescence detection by the appropriate secondary horseradish peroxidase-conjugated antibody.

Results

The IEC-6 cell growth as a function of time is presented in Figure 1. As shown, the exposure of the cells to gastrin, at a concentration of 250 ng/ml, causes a significant increase in cell growth in comparison to control cells. This increase is observed 6 hr after exposure to gastrin and is more apparent after 12 and 24 hr. Genistein, a specific inhibitor of tyrosine kinase, abolishes the effect of gastrin on cell growth without decreas-

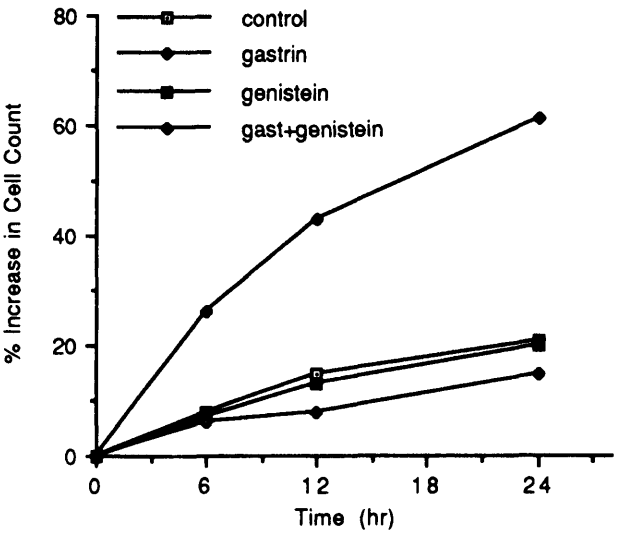


Figure 1. Effect of gastrin and genistein on cell proliferation. Cells 1.5×10^6 were seeded into parallel flasks and grown in DMEM (control), DMEM containing gastrin (250 ng/ml), DMEM containing genistein (100 μg/ml), and DMEM containing gastrin and genistein together. At the indicated time intervals, the cells were detached by trypsinization and counted. Values represent the average of three independent experiments.

ing the viability of the cells. In our studies, we used a 100-μg/ml concentration of genistein, which has been found to cause total inhibition of tyrosine kinase (9). The incubation of cells in the presence of genistein alone decreased, although not significantly, the rate of growth of IEC-6 cells when compared with the growth observed in the controls.

The interaction of sodium vanadate and gastrin was tested in the studies shown in Figure 2. Vanadate has been reported to inhibit phosphotyrosine phosphatase in different cell systems (13–15), and when it was placed in incubation media for 24 hr, it caused stimulation of cell growth that was equal to the stimulation caused by gastrin. The combination of gastrin and sodium vanadate caused a small but significant ($P < 0.01$) increase in cell growth over that observed by either of these two agents alone. This suggests that the two mitogenic agents potentiate each other. The dose response and time course of sodium vanadate on cell proliferation were tested and are shown in Figure 3. The maximal effect of sodium vanadate was observed at 0.08 m*M* (Fig. 3). A surprising finding, however, was the fact that genistein, as in the case of gastrin, eliminated almost completely the stimulation of cell growth that is caused by sodium vanadate (Fig. 4). This suggests that the effect of gastrin is through the activation of tyrosine kinase and that the mitogenic effect of sodium vanadate can occur only when tyrosine kinase activity is uninhibited.

Protein tyrosine phosphorylation in the intestinal cell line IEC-6 stimulated with gastrin was investigated

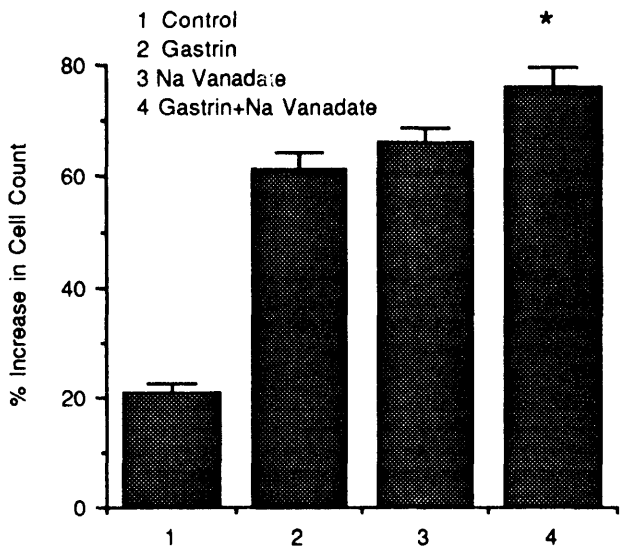
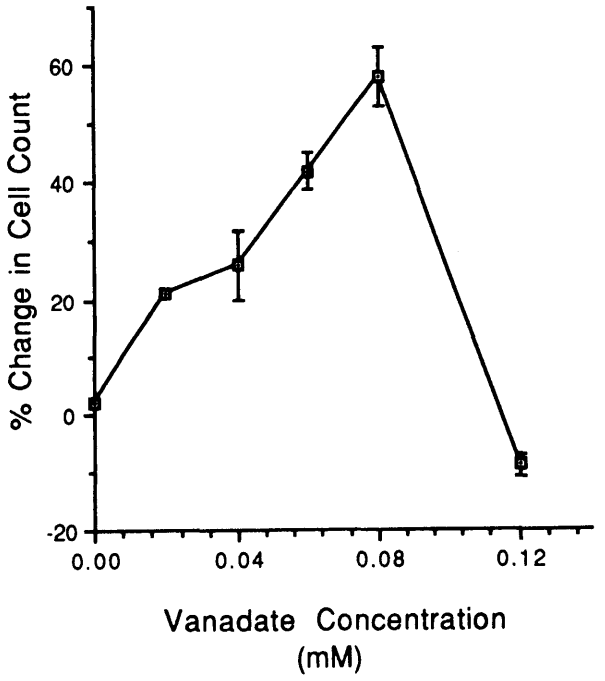


Figure 2. Effect of gastrin and sodium vanadate on growth of IEC-6 cells. Cells 1.5 × 10^6 were seeded into flasks containing DMEM alone, or gastrin (250 ng/ml), sodium vanadate (0.08 m*M*), or both gastrin and sodium vanadate together. At the end of 24 hr of incubation, the cells were detached by trypsinization and counted. Values are mean ± SE of six independent measurements. *$P < 0.01$ when compared with Conditions 2 or 3 (Student's *t* test).



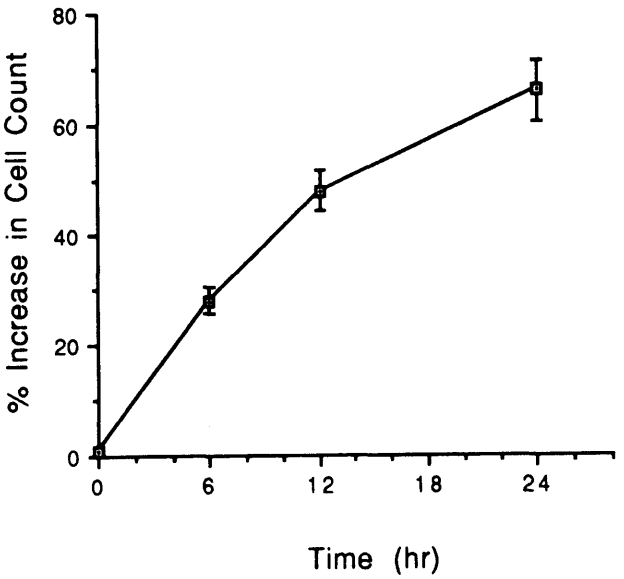


Figure 3. Cell proliferation as a function of sodium vanadate concentration and of time of incubation. In the dose-response curve, sodium vanadate was added to the incubation media in the concentrations indicated. A 0.08-m*M* vanadate concentration was used in the time course-study. Values are means of three independent measurements.

using antiphosphotyrosine antibody in Western blots. The addition of gastrin (250 ng/ml) to attached IEC-6 cells causes time-dependent increases in the levels of tyrosine phosphorylation in several proteins with molecular masses of 42, 45, 52, 60, 78, and 120 kDa (Fig. 5). The action of gastrin was rapid, and significant increases in the phosphorylation in several proteins can

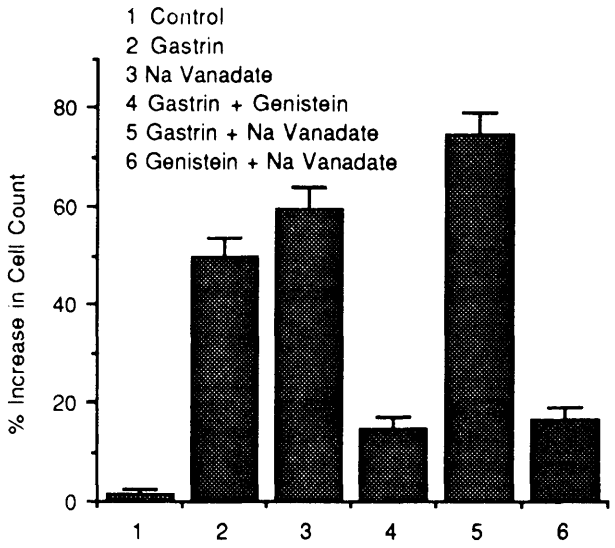


Figure 4. Cell proliferation in the presence and absence of gastrin, sodium vanadate and genistein. Cell counts in all conditions were done after 24 hr of incubation. Gastrin was added in a concentration of 250 ng/ml, sodium orthovanadate (0.08 m*M*), and genistein (100 μg/ml). Values are means of three independent determinations.

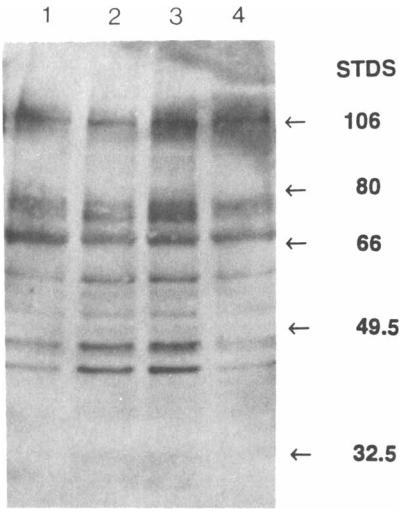


Figure 5. Phosphorylation of IEC-6 cell proteins after gastrin stimulation. The cells were stimulated with gastrin (250 ng/ml) for varying time periods. Lane 1 refers to control cells (without gastrin), and Lanes 2, 3, and 4 refer to stimulation with gastrin for 5, 10, and 15 min, respectively. Proteins were analyzed by sodium dodecyl sulfate with antiphosphotyrosine antibody followed by electrogenerated chemiluminescence detection. Several proteins showed an increase in the level of tyrosine phosphorylation at 5 min after gastrin exposure, reaching maximum at 10 min. By 15 min of treatment, the phosphorylation levels had decreased. Molecular masses of the standards are given in kDa. Densitometry values measured at 0, 5, 10, and 15 min were, respectively, 1.7, 3.3, 4.1, and 1.3 for the 120 kDa, 2.8, 3.7, 5.6, and 1.7 for the 78 kDa, 0.3, 0.9, 1.3, and 0.6 for the 60 kDa and 0.3, 0.6, 0.6, and 0.2 for the 42 kDa.

be seen as early as 5 min, reaching maximum at 10 min after gastrin addition. Also of note, is that the levels of gastrin-induced increases in phosphorylation of some proteins were transient. The levels of tyrosine phosphorylation in some proteins decreased after 15 min of incubation with gastrin. Tyrosine phosphorylation studies were also done 10 min after the addition of 0.08 m*M* sodium vanadate. Tyrosine phosphorylation levels of several proteins were increased (data not shown). Genistein inhibited the increases in phosphorylation produced by gastrin and vanadate (data not shown). As shown in Figure 6, we have also demonstrated, using Western blots, the presence of GTPase activating protein, raf1 kinase, phospholipase C γ-1, and phosphoinositide 3-kinase in IEC-6 cells.

Discussion

The finding in the present study of a stimulatory effect of gastrin on cell growth agrees with what has been described by Conteas and Majumdar (8), except that the rate of cell growth observed by us was about 25% higher than described previously. This difference is probably related to differences in the culture conditions and/or mode of gastrin administration. These authors measured thymidine uptake per unit protein, and the incorporation of thymidine into DNA correlated fairly well with estimates of cell growth as determined by hemocytometry. Our findings, however, as well as those of Conteas and Majumdar (8), do not agree with those of Baliga and Borowitz (16), who did not observe any effect of gastrin on cell growth or mitogenesis of IEC-6 cells. It is unclear at present, why such differences have been observed, but culture conditions, the number of passages of the crypt cells, and the presence or absence of fetal calf serum and/or insulin may all contribute to the differences observed by the different investigators. As shown in Figures 1 and 4, the observed stimulation by gastrin is inhibited by genistein, a known tyrosine kinase inhibitor (9). These findings provide evidence for concluding that the exposure of IEC-6 cells to gastrin causes stimulation of tyrosine kinase. A more compelling reason to conclude that gastrin promotes the stimulation of tyrosine kinase is the result of the studies shown in Figure 5, where the addition of gastrin produces a rapid increase in the

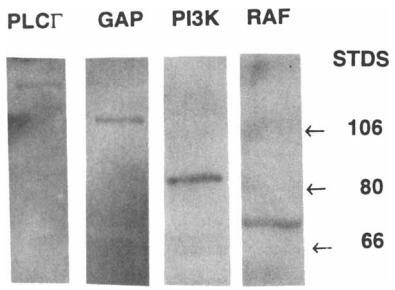


Figure 6. Proteins identified in normal IEC-6 cells. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blots were sequentially probed with antibodies to the indicated proteins followed by electrogenerated chemiluminescence detection. Molecular masses of the standards are given in kDa. The plot represents a 10-min time point.

phosphorylation on tyrosine of several proteins. The identities of these proteins are not known at present. There are several proteins whose phosphorylation on tyrosine residues is thought to be closely involved in the initiation of cell proliferation (17).

Another interesting finding in the present results is the observed stimulation of cell growth by sodium vanadate. Sodium orthovanadate stimulates growth by inhibiting phosphotyrosine phosphatases, an action that has been reported in different cell systems (13–15). In the present study, sodium vanadate appears to stimulates growth of IEC-6 cells by inhibition of phosphotyrosine phosphatases. In favor of this proposition is the fact that sodium vanadate potentiates the action of gastrin on cell growth by extending the phosphorylation effect through inhibition of the phosphatases. It is, however, unclear why genistein, which is a known tyrosine kinase inhibitor, also inhibits the trophic action of sodium vanadate in this cell system. It is possible that the vanadate effect only occurs when tyrosine kinase is uninhibited and protein phosphorylation undiminished. The role of vanadate could, therefore, be caused by a virtual increase in phosphorylation of cell proteins due to the reduced action of the phosphotyrosine phosphatases. A second explanation is that vanadate may, in itself, directly stimulate tyrosine kinase. There are studies that support this possibility (18–21), but further studies are clearly needed to identify which of these two mechanisms is involved.

The proteins whose phosphorylation on tyrosine is increased by gastrin have molecular weights that are close to those of proteins that are thought to be closely involved in proliferation in other cell types. Establishing that gastrin stimulates the phosphorylation on tyrosine of these proteins will increase our understanding of the signal-transduction mechanism of the gastrin-induced intestinal cell proliferation. For example, phosphorylation of mitogen-activated protein kinase on tyrosine activates this enzyme, which in turn phosphorylates several substrates, including c-*jun*, raf1 kinase, s6 kinase, and others. Determining the identities of these proteins using immunoprecipitation is the subject of future study.

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