Regulation of intestinal tyrosine phosphorylation and programmed cell death by peroxovanadate

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Scheving, Lawrence A., Jiji R. Thomas, and Linda Zhang. Regulation of intestinal tyrosine phosphorylation and programmed cell death by peroxovanadate. Am. J. Physiol. 277 (Cell Physiol. 46): C572–C579, 1999.—Cell suspensions of ileal mucosa undergo a rapid and synchronized form of programmed cell death when cultured in a simple medium at 37°C. Because tyrosine phosphorylation of proteins plays a crucial role in the signal transduction of many cellular processes, we examined its role in intestinal programmed cell death by use of immunoblot and immunohistochemical methods. We observed a 50–70% reduction in tyrosine phosphorylation during the initial 10 min of intestinal epithelial cell culture. We hypothesized that the inhibition of protein tyrosine phosphatases would increase protein tyrosine phosphorylation in these suspensions and decrease programmed cell death. A strong inhibitor of these phosphatases (peroxovanadate) but not a weaker one (sodium orthovanadate) abolished the DNA fragmentation/adhering normally seen in dying enterocytes. Peroxovanadate enhanced protein tyrosine phosphorylation of many intestinal proteins, dramatically increasing the dually phosphorylated and active form of mitogen-activated protein kinase. Immunohistochemistry revealed a particularly high level of increased tyrosine phosphorylation in the intestinal crypts in peroxovanadate-treated mucosa. Kinetic studies indicated that the pivotal time for protein tyrosine phosphatase inhibition occurred within 5 min of ex vivo culture, precisely when protein tyrosine phosphorylation declined. Our data suggest that tyrosine kinase inactivation or tyrosine phosphatase activation may initiate intestinal epithelial cell death.

activation of intracellular kinases by growth factors, hormones, and cytokines mediates many cell responses. Protein tyrosine kinases (PTKases) phosphorylate proteins on tyrosine residues. Protein tyrosine phosphorylation is a tightly regulated process, and the action of PTKases is counterbalanced by the ability of specific phosphatases, protein tyrosine phosphatases (PTPases), to dephosphorylate tyrosine. Indeed, specific phosphatase inhibitors, such as sodium vanadate, must be added to cell extracts to prevent tyrosine dephosphorylation during cell homogenization. Although the role of tyrosine phosphorylation in cell growth and differentiation has been extensively studied, very little is known about the role of phosphorylation in the regulation of programmed cell death (6, 7, 23, 25–27), which plays critical physiological and pathological roles in many tissues, including the small intestine (21).

The renewing epithelium of the small intestine has the highest rate of cell proliferation in the intact adult animal (20). To maintain its normal cell mass, the intestinal epithelium eliminates a large number of dying cells daily by releasing them into the intestinal lumen. Remarkably, the average human sheds his body weight in dying cells during a 6- to 9-mo period (14). In addition to regulating intestinal cell mass, apoptosis also plays an important role in intestinal injury, such as ischemic-reperfusion injury (5, 11) or intestinal barriers dysfunction (22). In contrast to the cell proliferation cycle, which extends over several hours and includes several well-defined stages, the study of intestinal programmed cell death in the intact animal is problematic for several reasons. The cell death pathway is short; some cells may detach from the intestinal villus early in the death pathway; and, finally, the biochemical and molecular stages of the cell death pathway are just being described.

We have recently reported that ex vivo cell suspensions of mouse intestinal epithelial cells in serum-free DMEM undergo massive, rapid, and highly synchronized programmed cell death (18). Similar models have been established using dissociated human intestinal epithelial cells (3, 4) and rat colonic crypt cells (7). These models synchronize cell death over a short time frame, allowing one to analyze the role of various signals transduction pathways in dying intestinal tissue.

In our original paper (18), we reported that dying enterocytes downregulate several tyrosine kinases and their modulators, including the epidermal growth factor receptor (EGFR), the Erb-B2 tyrosine kinase, and the son of sevenless (Sos) guanine nucleotide exchangers. This led us to speculate that the elimination of proteins involved in Ras-convergent signaling pathways may contribute to the initiation of apoptosis. We hypothesized that increased tyrosine phosphorylation achieved by inhibiting PTPase activity might prevent apoptosis. To examine the importance of protein tyrosine phosphorylation in intestinal epithelial cell death, we exposed cells to peroxovanadate, a potent and irreversible inhibitor of PTPases. This compound is generated by vanadate peroxidation in the presence of H2O2 and inhibits PTPases in excess to the sum of either vanadate or H2O2 (16). When injected in mice, peroxovanadate induces a striking increase in protein tyrosine phosphorylation in intact tissues.

In this report, we show that intestinal epithelial cell cultures undergo a rapid reduction in tyrosine phosphorylation within 10 min of culture at 37°C, preceding our previously reported downregulation in EGFR, Erb-B2,
Materials. B6D2F1 male mice were obtained from Jackson Laboratory (Bar Harbor, ME). Nitrocellulose was obtained from Micron Separation (Westboro, MA). Prestained protein standards were from Amersham Life Sciences (Arlington Heights, IL) or Bio-Rad Laboratories (Hercules, CA). The anti-phosphotyrosine antibodies for blotting (RC20H) and immunohistochemistry (pAb) were obtained from Transduction Laboratories (Lexington, KY). The rabbit polyclonal antibody against the dually phosphorylated MAP kinase was obtained from Promega (Madison, WI). Horseradish peroxidase-conjugated goat anti-rabbit antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence reagent was from New England Nuclear Life Science Products (Boston, MA). Sodium vanadate, and NaOH. The sample was neutralized as recommended with 1 M HEPES, and the DNA concentration was determined by spectrophotometric readings at 260 nm. Three micrograms of DNA were loaded per lane on a 2% agarose gel. The samples were electrophoresed for 2 h at 95 V, stained with ethidium bromide, transilluminated, and photographed.

Immunohistochemistry. Intestinal pieces or cell pellets were fixed in fresh 4% paraformaldehyde at 4°C for 4 h. Tissue pieces were then rinsed several times in 70% ethanol at 4°C, embedded in paraffin, and cut into 5-µm sections. Sections were processed by the manufacturer’s protocol (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was quenched by placing the slides in 0.3% H2O2 in methanol for 30 min. After blocking the slides in a 3.0% goat serum solution for 1 h, the tissue sections were incubated with rabbit anti-phosphotyrosine antibodies (0.5 µg/ml) in PBS for 90 min in a humidified chamber. The sections were then washed, incubated with biotinylated secondary antibody, rinsed in PBS, and incubated with the Vectastain EliteABC reagent. Diaminobenzidine was used as the peroxidase substrate.

RESULTS

Tyrosine phosphorylation declines rapidly in ileal cultures. To determine whether tyrosine phosphorylation of membrane and cytosolic proteins changed during ex vivo cell culture, we analyzed phosphorylation at various times within the first 15 min of cell culture in DMEM at 37°C. Cells were homogenized, separated into membrane and cytosolic fractions, and then resolved by SDS-PAGE. As shown in Fig. 1, immunoblot analysis of proteins (3 min chemiluminescent exposure) resolved on a 7% SDS polyacrylamide gel indi-
The ileal cell suspensions underwent massive programmed cell death which led us to hypothesize that inhibition of PTPases would reverse not only the reduction in tyrosine phosphorylation but also the initiation of programmed cell death. However, initial studies with a weak and reversible phosphatase inhibitor, sodium orthovanadate, at a concentration as high as 1 mM caused only small increases in tyrosine phosphorylation and did not prevent DNA fragmentation (data not shown). Additional studies were performed using a stronger and irreversible PTPase inhibitor, peroxovanadate (16). Intestinal epithelial mucosal suspensions were exposed to DMEM containing sodium vanadate, H$_2$O$_2$, or a mixture of sodium vanadate plus H$_2$O$_2$ (peroxovanadate). After 150 min, the intestinal cells were harvested and frozen in liquid nitrogen. Extracts were prepared and subjected to SDS-PAGE and transblot. The blots were exposed to anti-phosphotyrosine antibodies (RC20H). Preliminary experiments established that peroxovanadate but not sodium vanadate or H$_2$O$_2$ appreciably increased tyrosine phosphorylation of many proteins. A dose-response curve demonstrated the effect of varying concentrations of peroxovanadate on tyrosine phosphorylation (Fig. 2). Peroxovanadate even at the lowest concentration (60 µM) resulted in increased tyrosine phosphorylation (Fig. 2); however, the greatest increase occurred between 500 µM and 1 mM.

Peroxovanadate induces a rapid and sustained increase in tyrosine phosphorylation. To further characterize the action of PTPase inhibition, we carried out a time course for peroxovanadate (500 µM sodium vanadate and 8 mM H$_2$O$_2$). Maximum tyrosine phosphorylation occurred as early as 15 min of culture (Fig. 3A), but a pronounced increase was seen as early as 5 min and extended through the entire culture period. The time course of phosphorylation varied for individual proteins. For some proteins, maximal phosphorylation occurred at 15 min. For others, it occurred at later times between 90 and 150 min.

Peroxovanadate increases MAP kinase activation. Activation of MAP kinase requires phosphorylation of nearby threonine and tyrosine residues. Using an antibody that recognizes the dual phosphorylated (threonine/tyrosine) MAP kinase (1), we evaluated the effect of peroxovanadate on MAP kinase activation (Fig. 3B). We found that peroxovanadate not only reversed the decline of this enzyme normally seen in dying cells, but it also rapidly induced a sustained increase in MAP kinase activation.

The intestinal epithelial cell crypts show the most pronounced increase in tyrosine phosphorylation. The mucosal cell suspensions consisted of islands of cells derived from either villi (70–80%) or crypts (<20%). Preliminary immunohistochemical experiments using mucosal scrapings indicated that peroxovanadate disproportionately increased crypt cell phosphoryrosine staining. To better show this, we exposed a 1-cm piece of intact ileum (18) as well as mucosal suspensions to a peroxovanadate-containing (500 µM) medium for 150 min and then analyzed the cellular distribution of phosphotyrosine by immunohistochemistry using a rab-
bit anti-phosphotyrosine antibody (Fig. 4). These results confirmed that peroxovanadate caused a pronounced increase in tyrosine phosphorylation. They also showed a rather low level of phosphorylation in normal mouse small intestine. Notably, the crypts showed the most remarkable increase in tyrosine phosphorylation compared with the villi. Analysis of digital data on these slides using a Bioquant TCW-95 program (R & M Biometrics, Nashville, TN) and a Sony DXC-151A charge-coupled device camera indicated that the average signal in the peroxovanadate-treated crypts was ~100 times that in the villus.

Peroxovanadate treatment reduces the amount of DNA fragmentation in ex vivo intestinal epithelial cell cultures. We hypothesized that a reduction in PTPase activity would not only increase protein tyrosine phosphorylation in our cell cultures but would also decrease apoptosis. Indeed, the epithelium of the peroxovanadate-treated ileum had a more orderly appearance, with deeper crypts and longer villi compared with the

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**Fig. 2.** Exposure of intestinal cell cultures to peroxovanadate (PV) increases tyrosine phosphorylation in a dose-dependent manner. Intestinal cell cultures incubated in DMEM at 37°C were exposed to different concentrations of peroxovanadate. After 150 min, cells were pelleted, and the cell pellet was homogenized. Homogenates were mixed with Laemmli buffer, heated at 95°C for 5 min, loaded on 7% SDS-polyacrylamide gels, transblotted to nitrocellulose, and probed with anti-phosphotyrosine antibody (RC20H). Each lane contains 50 µg of membrane protein. Zero time represents cells exposed to ice-cold DMEM but immediately pelleted and homogenized. A: long chemiluminescent exposure (5 min). B: shorter exposure of same blot (2 min). Numbers at left are molecular mass standards (kDa).

**Fig. 3.** Peroxovanadate induces a time-dependent increase in tyrosine phosphorylation and in the dually phosphorylated form of active mitogen-activated protein (MAP) kinase. A: cells were exposed to 0.5 mM peroxovanadate for different lengths of time (+ PV). Cells were homogenized, and total homogenate fraction was prepared for immunoblot. Note that peak tyrosine phosphorylation was attained very early and persisted at the 150-min time point. Very little tyrosine phosphorylation was seen in cultures that were not treated with peroxovanadate (− PV). Numbers at left are molecular mass standards (kDa). B: proteins from intestinal cell cultures were immunoblotted and probed with an antibody that recognizes the dually phosphorylated form of MAP kinase. Note that while active MAP kinase decreases in − PV cultures, it dramatically increases in + PV cultures. Numbers at left identify the 2 MAP kinase forms (extracellular signal regulated kinases 1 and 2).
tegrated dying intestinal epithelium (Fig. 4 and data not shown). To further evaluate the influence of PTPase inhibition on apoptosis, we analyzed DNA laddering in treated mucosal suspensions. We have previously shown that the DNA from dying enterocytes on electrophoretic gels displays the laddered appearance characteristic of internucleosomal degradation of DNA, a hallmark feature of most forms of apoptosis (18). As shown in Fig. 5, DNA was purified from cells exposed to different concentrations of peroxovanadate and then resolved by electrophoresis on agarose gels. Peroxovanadate treatment decreased DNA fragmentation in a dose-dependent manner, correlating with the dose-response curve of protein tyrosine phosphorylation. In contrast, sodium vanadate (1 mM) or H2O2 (8 mM) had no appreciable effect on DNA fragmentation at the concentrations tested (data not shown).

Peroxovanadate treatment is required within the initial 5 min of cell culture at 37°C. Peroxovanadate caused a sustained increase in tyrosine phosphorylation in many proteins through the entire culture period. To determine whether the inhibitory effect of peroxovanadate on DNA fragmentation occurred at an early, intermediate, or late stage in the death pathway, cells were treated with peroxovanadate at varying times after the start of cell culture at 37°C (Fig. 6). This experiment showed that peroxovanadate effectively prevented DNA fragmentation when added at 0 or 5 min of culture but became ineffective at later times. Thus the essential PTPase inhibitory action in this

Fig. 4. Peroxovanadate (PV) causes a disproportionate increase in tyrosine phosphorylation in the intestinal crypts. Immunohistochemistry was done to localize the phosphotyrosine in histological sections from intact pieces of ileum [zero time (A), 150 min (B), and 150 min (C)] and in mucosal suspension cultures (150 min (D)). Very little signal was detected in sections from zero-time (A) piece or −PV (B) piece. In contrast, an intense signal was detected in sections of +PV piece (C) and +PV suspension cultures (D), with crypt and lower villus showing most intense signal. Bar in C designates crypt zone. Magnification for all, ×90.

Fig. 5. Peroxovanadate inhibition of protein tyrosine phosphatases (PTPases) leads to decreased DNA fragmentation. A: DNA was purified from detached mucosal cultures that had been exposed to increasing concentrations of peroxovanadate. Note that amount of DNA fragmentation decreases with increasing amounts of peroxovanadate. B: Fragmented DNA was quantified by laser densitometry.
model occurs within minutes of cell culture, coincident with the initial rapid decrease in protein tyrosine phosphorylation.

DISCUSSION

Protein tyrosine phosphorylation by transmembrane and cytoplasmic tyrosine kinases and dephosphorylation by PTPases have been shown to play prominent roles in cell function, particularly in the control of growth and differentiation. Less attention has been paid to the potential regulatory role of protein tyrosine phosphorylation and dephosphorylation in the regulation of programmed cell death. This may be partly attributable to the difficulty of synchronizing death in cell lines. We have developed a new and highly synchronous model of intestinal programmed cell death that uses mucosal cell suspension cultures in a serum-free medium (18). Using this model, we found that the overall level of protein tyrosine phosphorylation rapidly decreased within minutes of incubation in DMEM at 37°C (Fig. 1).

Whereas many membrane and cytosolic proteins showed a significant decrease in their phosphotyrosine content during the first 10–15 min of culture, two prominently phosphorylated membrane proteins showed an extremely rapid apparent loss of phosphotyrosine between 2.5 and 5 min. The identity of these proteins, which are estimated to be 87 and 90 kDa, is unknown. We originally speculated that the 87-kDa protein was the regulatory subunit of phosphatidylinositol 3-kinase, which has been implicated in the regulation of programmed cell death (6, 23, 26); however, the position of this protein did not precisely correlate with phosphatidylinositol 3-kinase by immunoblot (data not shown). Whether the loss of phosphotyrosine for the 87- and 90-kDa proteins is due to an abrupt decrease in a specific PTKase activity or an increase in PTPase activity or both is not known. Other explanations for the disappearance of signal are also possible. The apparent loss in phosphotyrosine could be mediated by proteolytic degradation or by translocation from the membrane to the cytosol. The latter possibility seems unlikely, however, because the loss of signal at the 87- and 90-kDa positions in the membrane fraction was not accompanied by a corresponding increase of signal in the cytoplasmic fraction.

Our experiments underscore the potential importance of PTPase activity in either the regulation or possible therapeutic prevention of programmed cell death. Exposure of intestinal epithelial cells to a vanadate/H₂O₂ mixture resulted in the rapid and sustained appearance of many tyrosine-phosphorylated proteins (Figs. 2 and 3), including the 87- and 90-kDa proteins noted above. It also increased the dually phosphorylated and active form of the important intracellular regulatory protein MAP kinase (Fig. 3B), which in some cell systems has an anti-apoptotic role (10, 27) and which also became dephosphorylated during cell death. Although an immunohistochemical analysis revealed increased tyrosine phosphorylation in both crypt and villus compartments, it was more pronounced in the crypt (Fig. 4). This may reflect a higher basal level of PTKase activity or a lower basal level of PTPase activity in the crypt than the villus.

Although peroxovanadate inhibited programmed cell death, sodium vanadate and H₂O₂ had little or no independent action on death at the concentrations tested. Yet, in related recent work, other investigators have presented convincing histochemical evidence that 1 mM vanadate inhibits programmed cell death of isolated rat colonic crypt cells (7). The requirement for a more potent PTPase inhibitor to inhibit programmed cell death in the small intestine suggests that it may have a higher level of endogenous PTPase activity than the colon. If so, this may contribute to the resistance of the ileum compared with the colon to neoplastic transformation.

Peroxovanadate preserved the histological appearance of the cultured intestine, which more closely resembled the normal intestine (Fig. 4 and data not shown). Peroxovanadate also decreased DNA fragmentation in cell cultures, provided it was added within the initial 5 min of cell culture at 37°C (Figs. 5 and 6). When peroxovanadate was added at later times, DNA fragmentation could not be prevented. Because significant DNA fragmentation does not manifest until 60–90 min of cell culture, this experiment suggests that the antiapoptotic action of PTPase inhibition by peroxovanadate occurred shortly after exposure to culture medium and that the rapid tyrosine dephosphorylation seen...
within the first 5 min of culture may be a critical step in the cellular progression to death.

If so, then the tyrosine phosphorylation/dephosphorylation steps required for cell survival may occur in the initiation phase of programmed cell death as opposed to the later commitment and execution phases. The latter phases involve alterations in mitochondria, leakage of cytochrome c, and activation of caspases and DNA fragmentation factors. Much less is known about the initiation phase, which is associated with cell surface proteins and membrane events such as membrane blebbing. Because the disruption of cell-cell and cell-basement membrane contacts normally occurs as intestinal epithelial cells die and exfoliate, the phosphatase-mediated dephosphorylation of proteins involved in cell-cell or cell-basement membrane contacts may initiate a death program in our suspension cultures.

Focal adhesion kinase (FAK) and EGFR are two candidate PTPase targets that play an important role in the maintenance of tissue integrity and may be involved in the progression of intestinal apoptosis. Both proteins have been shown to be regulated by PTPases (12, 15). The interaction of extracellular matrix proteins and integrins can activate FAK, which is a cytoplasmic tyrosine kinase implicated in integrin-mediated signal transduction pathways. During integrin-mediated cell adhesion, FAK in surviving cells becomes activated, tyrosine phosphorylated, and colocalized to focal contacts with integrins and other cytoskeletal proteins. During endothelial cell apoptosis, FAK became dephosphorylated on tyrosine residues (2, 8, 15, 27). Moreover, constitutively activated forms of FAK rescued two established epithelial cell lines from anoikis-type apoptotic death (2).

A potential role for the EGFR and other Erb-related proteins in enterocyte adherence, migration, and death is of particular interest, since these proteins have no clear function in postmitotic intestinal epithelial cells but are expressed at a relatively high level (13, 19). Recently, integrins have been shown to physically associate with EGFR and to cause it to autophosphorylate in a ligand-independent manner as part of a novel signaling pathway to promote adhesion-dependent cell survival (10). Moreover, in some tumor cells, EGFR kinase blockade by monoclonal antibodies or pharmacologic inhibitors causes apoptosis (9). In other tumor cells, an early cell surface event in the death pathway induced by tumor necrosis factor-α was EGFR dephosphorylation by PTPase 1B (12).

By inhibiting PTPases, peroxovanadate globally increases tyrosine phosphorylation of cell surface receptors, cytoplasmic and nuclear proteins, even in the absence of receptor tyrosine kinase-activating ligands (16). The present report suggests that PTPases may play an important role in the positive regulation of programmed cell death of intestinal epithelial cells. Although the role of PTPases in this process has not received much attention, the forced overexpression of the leukocyte common antigen-related (LAR) phosphatase has recently been shown to cause apoptosis in 3T3 cells (24). We have found a high expression level of this transmembrane tyrosine phosphatase in the ileum (data not shown). Thus it will be of interest to determine whether LAR or other membrane-bound or cytosolic PTPases are activated or stabilized during the initiation phase of cell death and to determine to what extent the death pathways in the replicating crypt and postmitotic villus cells differ from each other and from various apoptotic pathways currently being defined in other models.

This work was supported by Ross Products Division-Abbott Laboratories (BE’97) and National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-45925.

Received 10 February 1999; accepted in final form 27 April 1999.

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