Anti-apoptotic Signaling of Pleiotrophin through Its Receptor, Anaplastic Lymphoma Kinase*

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The secreted growth factor pleiotrophin (PTN) can induce mitogenesis in cells that express the receptor for this growth factor, anaplastic lymphoma kinase (ALK). Here we examine the ability of PTN to produce anti-apoptotic signals. We demonstrate that PTN is a survival factor for SW-13 epithelial cells and show that ribozyme-mediated depletion of ALK from SW-13 cells abolishes this effect of PTN. Furthermore, in serum-starved NIH3T3 fibroblasts PTN prevents apoptosis (measured by annexin V staining) with an EC50 of 0.2 ng/ml and induces cell growth at higher concentrations of PTN. A polyclonal antibody against the PTN ligand-binding domain of the ALK receptor (α-LBD) was a partial agonist for ALK in NIH3T3 cells. This α-LBD antibody showed high agonist activity for anti-apoptosis (56 ± 9% relative to PTN), low agonist activity for cell growth (21 ± 1% relative to PTN), and was an antagonist of PTN-induced cell growth (61 ± 2% inhibition). Both MAP kinase and phosphatidylinositol (PI) 3-kinase cascades in NIH3T3 cells were activated by PTN, and this effect persisted for up to 3 h. Surprisingly, the anti-apoptotic effect of PTN was completely blocked by the MAP kinase inhibitor U0126, but was not affected by the PI 3-kinase inhibitor LY294002. In contrast, PTN-dependent cell growth required both MAPK and PI 3-kinase activity. We conclude that anti-apoptotic signaling of PTN through ALK in NIH3T3 fibroblasts is via the MAP kinase pathway.

The ability of multicellular organisms to maintain homeostasis is dependent upon a delicate balance between cell survival and programmed cell death or apoptosis. One of the key elements regulating this balance is growth factor signaling. Growth factors and cytokines have been shown to regulate a wide variety of cellular effects, including cell survival. Growth factor-mediated effects occur through the stimulation of specific intracellular signaling cascades via the intrinsic tyrosine kinase activities of their receptors. For tyrosine kinase receptors, ligand-dependent activation results in autophosphorylation of tyrosine residues within the cytosolic domain of the receptor creating specific binding sites for intracellular signal transduction molecules.

A number of growth factors, including insulin-like growth factor I, epidermal growth factor, basic fibroblast growth factor, platelet-derived growth factor, nerve growth factor, and cytokines such as interleukin-3 promote cell survival through pathways requiring the activation of PI1 3-kinase for review, see Ref. 1. The lipid products of PI 3-kinase act as second messengers to stimulate the activity of the serine/threonine kinase Akt (for review, see Ref. 2). Activated Akt has been shown to phosphorylate the pro-apoptotic Bad protein on serine 136, resulting in its dissociation from a complex with Bcl-xl (3). The uncomplexed Bcl-xl can suppress cell death responses by blocking the release of mitochondrial cytochrome c, a critical step in the activation of the downstream caspase cascade, required for apoptosis (4). Recent reports suggest that Bad can also be phosphorylated in a MAPK-dependent manner at serine 112 (5). This event also will disrupt the association of Bad with the Bcl-2/Bcl-xl complex and inhibit apoptotic signaling (5). Therefore, activation of either PI 3-kinase or Ras/Raf/MAPK can lead to the inhibition of apoptosis in response to growth factor stimuli.

Pleiotrophin (PTN) is an 18-kDa heparin-binding growth factor named for its pleiotrophic effects (for review, see Ref. 6). The described biological consequences for PTN-mediated signaling include neurite outgrowth, angiogenesis, and mitogenesis of fibroblast, endothelial, epithelial, and some tumor cell lines. We have previously reported that PTN can induce the activation of both the MAPK and PI 3-kinase pathways in bovine epithelial lens (BEL) cells, and we have also demonstrated a direct role for PTN in mitogenesis (7). In fact, most of the published literature on PTN has described the role of this growth factor within the context of cell growth and proliferation (8–12). Here we will address the role of PTN in a complementary cellular response, the regulation of survival and apoptosis.

Our laboratory recently identified a tyrosine kinase receptor for PTN, the anaplastic lymphoma kinase (ALK) (13). Published literature on the tyrosine kinase portion of this receptor was generated originally from clinical studies where a t (2:5) chromosomal translocation resulted in the fusion of the cytosolic, tyrosine kinase domain of ALK with a nuclear protein, nucleophosmin (NPM) (14, 15). This NPM-ALK fusion protein homodimerizes, resulting in constitutive activation of tyrosine kinase activity (16). This translocation is present in more than half of the anaplastic large cell lymphomas, a subgroup of non-Hodgkins lymphomas, and under these conditions NPM-ALK is oncogenic (14).
In the present study we examine PTN-dependent signal transduction via the tyrosine kinase growth factor receptor ALK. We show a concentration-dependent inhibition of apoptosis for NIH3T3 fibroblasts in response to PTN, and we demonstrate that this response is mediated by ALK, the receptor for PTN. This effect is largely due to activation of the MAPK pathway and is independent of the PI 3-kinase pathway. However, at least in NIH3T3 cells, stimulation of PI 3-kinase is involved in mitogenesis, suggesting that both of these pathways are utilized in response to ALK based signaling events.

EXPERIMENTAL PROCEDURES

Materials—The rabbit polyclonal anti-ALK ligand-binding domain (LBD) antibody was described recently (13). In brief, antiserum was raised in rabbits against a fusion protein between glutathione S-transferase and the LBD of pleiotrophin in the ALK receptor extracellular domain. This antiserum was affinity purified, and the resulting anti-LBD antibody was shown to block binding of radiolabeled PTN to the ALK receptor in intact cells (13). All commercially available primary antibodies were from New England Biolabs (Beverly, MA) except for the polyclonal anti-phosphotyrosine (Upstate Biotechnology Inc., Lake Placid, NY). The secondary anti-mouse and anti-rabbit horseradish peroxidase-conjugated antibodies were from Amersham Biosciences. Lysates were fractionated by SDS-PAGE on 10% gels and analyzed by Western blot analysis using antibodies as indicated.

Cell Lysates and Immunoblotting—For total protein lysates, NIH3T3 cells were seeded at 1.4 × 10⁴ per 10-cm dish in serum-containing medium. Cells were left to settle and spread overnight. After washing twice with PBS (150 mM NaCl, 1.9 mM Na₂HPO₄, pH 7.2), serum-free medium was added, and the cells were serum-starved for 18–24 h. Cells were treated with various doses of PTN for the times indicated. At each time and dose point, cells were washed twice with ice-cold TBS (150 mM NaCl, 50 mM Tris/HCl, 50 mM Tris, pH 7.5). 0.5 ml of lysis buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 0.05% deoxycholate, 1% Nonidet P-40, 1 mM vanadate, 50 mM NaF) supplemented with Complete™, a protease inhibitor mixture (Roche Molecular Biochemicals), was then added to each plate. After a short incubation at 4 °C, cell bodies were sonicated to the lysis buffer. Samples were briefly sonicated and cell debris removed by centrifugation at 10,000 × g for 10 min at 4 °C. The protein concentration of each sample was determined using the BCA assay (Pierce). For SDS-PAGE and Western blotting 10 μg of each sample was used per lane of a 4–20% gradient gel (Invitrogen). Proteins were electro-transferred to nitrocellulose, nonspecific sites were blocked with 3% bovine serum albumin in PBS, and blots were probed. For immunoprecipitation experiments, 500 μg of each lysate was incubated with a 15-μl suspension of protein A-Sepharose beads (Roche Molecular Biochemicals) for 2 h at 4 °C. After this preclaring step, 2 μg of a polyclonal anti-phosphotyrosine antibody and a 15-μl suspension of protein A-Sepharose beads were incubated with the lysates for 2 h at 4 °C. Beads were washed six times in immunoprecipitation buffer and proteins eluted from the beads by boiling in SDS-PAGE sample buffer under reducing conditions. Protein immunocomplexes were separated by SDS-PAGE on 10% gels and analyzed by Western blot analysis using antibodies as indicated.

Data Analysis—The Graphpad Prism 3.0 software was used for data analysis. t tests were applied for single comparisons and analysis of variance for multiple comparisons. p values <0.05 were considered statistically significant.

RESULTS

PTN Promotes Cell Survival—We have previously described the cell growth effects of PTN for a number of normal and tumorigenic cell lines. In particular, we have demonstrated that PTN induced colony formation and tumorigenicity of human SW-13 adrenal carcinoma cells (12) and overexpression of the PTN receptor ALK enhanced the effect of the growth factor on colony formation of these cells (13). SW-13 cells do not express PTN or the related growth factor midkine (6), but they express endogenous ALK, and ALK is phosphorylated rapidly in response to exogenously added PTN (13). We used this cell line as a first model to study the contributions of PTN signaling through ALK to cell growth and survival. To assess the significance of ALK for the PTN effect, we depleted the endogenous ALK from these cells using ALK-targeted ribozymes as described recently for U87 glioblastoma cells (18) and monitored the response of the cells to PTN. PTN stimulates colony formation of SW-13 cells in soft agar (Fig. 1a; see also Ref. 19) and stable pools of SW-13 cells expressing different ALK-targeted ribozymes (ALK Rz cells) failed to respond to PTN (Fig. 1a). It is noteworthy that colony formation in response to FGF-2 was not altered significantly in the ALK Rz cells in comparison with control cells (data not shown), supporting the specificity of the ribozyme targeting. This ALK-dependent PTN-induced colony formation of SW-13 cells corroborates our earlier findings that showed a strict relationship between PTN signal transduction
and residual ALK levels in U87 glioblastoma cells after ALK depletion by ribozymes (18). Furthermore, lack of a growth response of SW-13/ALK Rz cells to PTN supports the notion of ALK as the rate-limiting transmembrane receptor for growth signal transduction of PTN.

Since many cell lines undergo apoptosis upon serum starvation, we used this deprivation from a growth stimulus to examine to what extent the PTN/ALK axis contributes to cell survival of SW-13 cells. We compared cell survival of control and ALK Rz cells and found equivalent numbers of surviving cells per field when serum was present (Fig. 1c). In the absence of serum, both cell lines showed a significant reduction in the numbers of surviving cells, and PTN at 1 ng/ml was only able to rescue the control, but not the ALK Rz cells (Fig. 1, b and c). These data show that PTN positively modulates the cell survival of SW-13 cells via signaling through its receptor, ALK.

Since the cell growth effects of PTN have been described for a variety of cell lines, including fibroblasts, endothelial, and tumor cell lines (12), we used an additional model, NIH3T3 fibroblasts, to analyze the survival effects of PTN and dissect the signaling pathway further. Earlier studies showed that NIH3T3 cells are sensitive to PTN-induced mitogenesis, and overexpression of PTN in these cells has been shown to induce a transformed phenotype, including tumorigenicity and tumor metastasis in nude mice (20). We reported that NIH3T3 cells express ALK (13), and we reasoned that a comparison of the signaling toward proliferative and anti-apoptotic responses of these cells to PTN would reveal the underlying pathways. First, we confirmed that serum withdrawal induced significant apoptosis. We found that 18 h of serum starvation induced an increase from below 5% of cells to over 50% of cells in apoptosis, and representative data from a typical experiment are shown in Fig. 2a. Inclusion of PTN prevented apoptosis (Fig. 2a, -serum versus +PTN), and we characterized this effect as concentration-dependent (Fig. 2b). We found that the maximal response was reached at a PTN concentration of 1.0 ng/ml with an approximate EC<sub>50</sub> of 0.2 ng/ml (= 12 pm). Serum served as a positive control and conditioned media from control cells as a negative control (Fig. 2b, filled triangle and open circle). In parallel with the anti-apoptotic response of NIH3T3 cells to PTN, we also characterized the growth response (Fig. 2c). Whereas the EC<sub>50</sub> of PTN for anti-apoptotic effects was ~0.2 ng/ml, this concentration was barely effective on proliferation (compare Fig. 2, b and c), and we estimate a >5-fold difference in the sensitivity of these two cellular responses, i.e. survival and proliferation to PTN.

As a next step in the analysis, we used a polyclonal rabbit antibody raised against the LBD of ALK (α-LBD) to evaluate the contribution of ALK to the PTN effects. This antibody was shown earlier to block the binding of PTN to the ALK receptor in intact cells (13), and we tested this antibody for its activity profile in the cell growth assays. It has been shown that the binding of antibodies to the extracellular domain of tyrosine kinase receptors can induce partial agonist activity (21) due to the dimerization of the receptor ECD, e.g. the anti-HER-2 antibody 4D5 (22) has become one of the best known examples due to the clinical utility of the humanized antibody in the treatment of breast cancer (23). Furthermore, different intrinsic activities for different cellular responses have been reported for a given antibody targeting a specific receptor pathway, e.g. the hepatocyte growth factor receptor c-Met (24) or the insulin receptor (25). Interestingly, we found a relatively low partial agonist
Fig. 2. Effect of PTN and α-LBD ALK antibody in NIH3T3 cells. a, fluorescence-activated cell sorter analysis for annexin V and propidium iodide staining of NIH3T3 cells. Analysis of cells in the presence of serum (left), after 18 h of serum starvation in the absence (middle), and in the presence of 1 ng/ml PTN (right) is shown. The percentages give the portion of cells in the respective quadrant. FITC, fluorescein isothiocyanate; b, quantitation of apoptosis using annexin V staining. The effect of different concentrations of PTN and polyclonal α-LBD antibody is shown. 10% serum served as a positive control and conditioned media from SW-13 cells (open circle) or a control antibody (open triangle) as a negative control. The data are means from at least five sets of experiments (± S.E.) as exemplified in a. The portion of cells displaying high annexin V staining after serum starvation (lower right quadrant in the fluorescence-activated cell sorter analysis) was used as the respective internal control for each experiment and set as 100%. c, cell growth. The effect of different concentrations of PTN and of the α-LBD antibody are shown with a control antibody (open triangle) as a negative control. Cell growth was measured using a colorimetric assay (see “Experimental Procedures”). Inset, effect of PTN (2 ng/ml), α-LBD (100 μg/ml), and PTN + α-LBD on cell growth. **, p < 0.001 relative to control or α-LBD or PTN + α-LBD. #, p > 0.05 between the two treatments and relative to control (analysis of variance).
activity of the α-LBD for proliferative effects (21 + 1% relative to PTN = 100%; Fig. 2c). On the other hand, addition of the α-LBD together with a concentration of PTN that stimulates proliferation above the intrinsic activity of the α-LBD antagonized the PTN response by 61 + 2% to a level indistinguishable from that reached by the α-LBD alone (Fig. 2c, inset). These findings are consistent with the partial agonist activity of the α-LBD antibody and support a functional significance of the α-LBD competition for PTN ALK receptor binding reported earlier (13). Furthermore, these data provide direct evidence that ALK is a receptor for PTN not only in SW13 and U87 cells, as we have already demonstrated, but is also the receptor for PTN in NIH3T3 cells (13, 18). It should be noted that the concentrations of α-LBD antibody give the total amount of IgG that was purified from rabbit antiserum and control purified IgG used at the same concentrations had no effect (Fig. 2, b and c, open triangle).

As described above (Fig. 2, b and c), we observed a >5-fold higher sensitivity of the anti-apoptotic versus the growth effect of PTN. We hypothesized that this difference might also be reflected in a higher agonist activity of the α-LBD antibody effect on apoptosis. Indeed, the α-LBD antibody showed a concentration-dependent inhibition of apoptosis of serum-starved NIH3T3 cells with a maximal activity of 56 + 9% relative to PTN, the full agonist (Fig. 2b). This is significantly higher than the 21 + 1% intrinsic activity observed for the growth effect (p < 0.005; Fig. 2c). However, the EC₅₀ of the antibody for the two effects is indistinguishable.

PTN Stimulates Both PI 3-Kinase and MAP Kinase Cascades in NIH3T3 Cells—Previously published work using mutant receptor constructs of the insulin-like growth factor I receptor and inhibitors of PI 3-kinase have suggested that PI 3-kinase is a major factor for the transduction of cell survival signals. Later work identified the serine/threonine kinase Akt as a downstream target of PI 3-kinase that in turn will phosphorylate and thereby inactivate the pro-apoptotic proteins BAD, caspase 9, and PKHR1L. We demonstrated previously that PTN signaling will elicit activation of both MAPK and PI 3-kinase in BEL cells (7), but only PI 3-kinase in U87 glioblastoma cells due to a constitutive activation of MAPK in those cells (18). Thus, we initially sought to characterize the activation of these pathways in the NIH3T3 model. We isolated cell lysates from NIH3T3 cells treated with PTN and performed a time course to examine activation of both the MAPK and PI 3-kinase pathways. MAPK activation was measured using phospho-specific antibodies, and the time course showed that MAPK was fully activated by 5 min of exposure to 1.0 ng/ml PTN and that this activation was sustained for up to 3 h (Fig. 3a). To examine PI 3-kinase activation we tested phosphorylation of both Akt and the p85 subunit of PI 3-kinase. To confirm that Akt was activated in parallel with PI 3-kinase, phosphorylated proteins were immunoprecipitated, and p85 was detected by Western blotting. Both p85 and Akt were maximally phosphorylated by 5 min of exposure to PTN, and this activation was also sustained over the entire 3-h time course (Fig. 3b). This time course of PTN activation of PI 3-kinase was similar to that observed in U87 cells (18). We also tested for ligand-dependent phosphorylation of endogenous ALK in the NIH3T3 cells but were unable to detect a clear signal due to the low level of endogenous receptor in these cells. This was similar to an only small induction of ALK phosphorylation by PTN in SW-13 cells (13) and by the PTN-related growth factor midkine in WI-38 human fibroblasts.²

PTN Inhibition of Apoptosis in NIH3T3 Cells Is Mediated by ²Stoica, G. E., Kuo, A., Powers, C., Bowden, E. T., Buchert-Sale, E., Kiegel, A. T., and Wellstein, A. (2002) J. Biol. Chem. DOI10.1074jbc.M205749200.
on the fusion of the tyrosine kinase domain of ALK to NPM as the result of a t (2:5) chromosomal translocation (26). This fusion results in a protein with transforming potential (27) able to produce an anti-apoptotic signal via constitutive PI 3-kinase activation (28). Studies regarding the characterization of this fusion protein have revealed a number of signaling cascades that exhibit constitutive activation due to the presence of this protein. In particular the adaptor proteins Shc, Grb2, and IRS-1 are found in association with NPM-ALK. Also phospho-protein. In particular the adaptor proteins Shc, Grb2, and IRS-1 are found in association with NPM-ALK. Also phospho-

We recently identified ALK as the tyrosine kinase receptor for PTN. In this study we also demonstrated that ALK is rapidly phosphorylated in response to PTN as early as 1 min after addition of the ligand (13). Furthermore, we showed blockade of ALK receptor binding by α-LBD antibodies, by added ALK-ECD protein or by anti-PTN antibodies, and we demonstrated that receptor phosphorylation and downstream signaling is inhibited by anti-PTN antibodies as well as by the ALK-ECD. We were unable to demonstrate a direct phosphorylation of ALK in NIH3T3 cells due to low levels of endogenous receptor in these cells but have demonstrated ALK phosphorylation in response to PTN in SW-13 cells (13) and recently in response to midkine in WI-38 human fibroblasts.2

In the present studies we used the α-LBD antibody as a tool to delineate PTN interaction with the ALK receptor in NIH3T3 cells. This polyclonal antibody acts as a partial agonist and stimulates proliferation 21% and inhibits apoptosis 56% relative to PTN (∼100%). This kind of distinct partial agonist activity for different cellular responses was also reported previously for antibodies interacting with c-Met (24) or the insulin receptor (25) and is likely due to a different extent of coupling of the respective downstream cascade to the activation by the receptor (21). The lower threshold concentration and EC50 of PTN for the anti-apoptotic versus proliferation effect (see Fig. 2, a versus b) already indicated a higher sensitivity and thus better coupling of the receptor to the downstream signal, and the differential intrinsic activity of the partially agonistic antibody corroborates this. It is noteworthy that the α-LBD antibody inhibits stimulation of proliferation by PTN (Fig. 2c, inset) providing additional evidence that ALK is the receptor for PTN in NIH3T3 cells.

As we pursued the downstream signaling toward proliferative and anti-apoptotic effects further in this model, we found, surprisingly, that MAP kinase and not the expected PI 3-kinase activation was the rate-limiting survival signal of PTN in these cells. Only few studies have suggested that MAPK stimulation can mediate an anti-apoptotic effect independently of the PI 3-kinase-Akt axis (32, 33). These studies demonstrated that two independent growth factors, although stimulating the same pathways in the same cell type, differed in terms of their ability to induce proliferation and anti-apoptotic signals; while both interleukin-3 and granulocyte macrophage-colony-stimulating factor can stimulate PI 3-kinase and Akt activation, the anti-apoptotic effect of granulocyte macrophage-colony-stimulating factor on hematopoietic cells was completely dependent upon the MAPK axis, whereas a growth response required activation of both MAPK and PI 3-kinase (33). Fang et al. (32) demonstrated that LPA stimulation of fibroblast cell lines, including NIH3T3, resulted in stimulation of both the PI 3-kinase and MAPK pathways. In that study, similar to our present report for PTN, anti-apoptotic signals were mediated predominantly through MAPK. However, activation of both cascades was necessary for cell growth. They also suggest that the co-operation of different signals was required for a proliferative response. Furthermore, they suggest that the survival function of LPA can be dissociated from its mitogenic activity. We propose that this is also true for PTN signaling via ALK. Hence, the role of MAPK as an anti-apoptotic mechanism is dependent not only on the cell type used, but also is somewhat specific to the receptor that triggers the stimulation. In fact this is the first report for an anti-apoptotic signal via a tyrosine kinase receptor mediated entirely by the MAPK pathway. Importantly, the dominance of this pathway modulating apoptosis is presented with data suggesting that the usual anti-apoptotic axis of PI 3-kinase/Akt is activated yet is not utilized for cell

\[ \text{Fig. 4. Effect of inhibitors on the PTN response in NIH3T3 cells.}\]

\[ \text{Concentration response of PTN on apoptosis (a) and on cell growth (b) in the absence (open circles) and in the presence of the MAPK inhibitor UO126 (closed circles) or the PI 3-kinase inhibitor LY294002 (closed triangles). Apoptosis was induced by serum starvation and was quantitated by annexin V staining and analyzed as shown in Fig. 2. Cell growth was measured by colorimetric assay (details under "Experimental Procedures").} \]
3-kinase activation is critical for PTN-induced cytoskeletal reorganization and Akt activation. In fact, in a parallel study we found that PI 3-kinase is activated downstream of the receptor tyrosine phosphatase RPTP-α that is utilized for PTN signal transduction. Our present findings are in contrast to the extensive published literature describing growth factors that promote cell survival through pathways requiring the activation of PI 3-kinase and Akt (1, 2). Activated Akt has been shown to phosphorylate Bad on serine 136, resulting in its dissociation from a complex with Bcl-xl (3). The uncomplexed Bcl-xl can suppress cell death responses by blocking the release of mitochondrial cytochrome c (4). However, recent reports suggest that Bad can also be phosphorylated in a MAPK-dependent manner at serine 112 (5). It has been demonstrated that serine phosphorylation at either of these positions will lead to dissociation of BAD from Bcl-xl. Therefore either the MAPK or the PI 3-kinase cascades have the potential to inhibit an apoptotic response via the same target molecules.

In summary, we show that PTN rescues both SW-13 and NIH3T3 cells from serum starvation-induced apoptosis, and we provide evidence that this effect is via the endogenous ALK receptor in these cells. We show in NIH3T3 cells that PTN induces MAPK as well as PI 3-kinase activity and find that PTN-induced growth requires both pathways, whereas anti-apoptotic effects of the growth factor are only dependent on MAPK and not on the PI 3-kinase-Akt axis.

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