PTEN is a negative regulator of STAT3 activation in human papillomavirus-infected cells

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Laryngeal papillomas are caused by infection of the laryngeal epithelium by human papillomavirus type 6 or type 11 (HPV-6/-11). Previous studies in our laboratory have demonstrated an increase in PI3 kinase levels in papilloma tissue. However, activation of the downstream effector of PI3 kinase, protein kinase B (PKB/Akt), was reduced. This observation was explained by the elevated expression of the phosphatase and tensin homologue (PTEN), a recently characterized tumour suppressor, in papilloma tissue. Recent investigation of the possible functional roles of PTEN during papilloma development has now indicated that the level of tyrosine(705)-phosphorylated signal transducer and activator of transcription 3 [PTyr(705)STAT3] could be inversely correlated to that of PTEN as well. In vitro phosphatase assays suggested the presence of an increased level of a PTyr(705)STAT3 phosphatase in papilloma extract. Immunodepletion of PTEN from papilloma extracts resulted in a reduction of the PTyr(705)STAT3 phosphatase activity. Transfection of PTEN cDNA into HeLa cells attenuated STAT3 phosphorylation at Tyr(705) in a dose-dependent manner. This attenuation of STAT3 phosphorylation was independent of the STAT3 kinase. Interestingly, introduction of a lipid phosphatase mutant of PTEN (G129E) resulted in heightened PTyr(705)STAT3 phosphatase activity, relative to that obtained from wild-type PTEN transfection. These data indicate that PTEN negatively regulates STAT3 activation in HPV-infected papilloma cells. Induction of PTEN and reduction of activated STAT3 might be a result of a host defence mechanism or a virus-directed strategy to alter normal epithelial differentiation programming.

Introduction

Human laryngeal papillomas are benign tumours of the respiratory tract that are due mainly to infection by human papilloma virus type 6 or type 11 (HPV-6/-11). Previous studies in this laboratory have shown that the level of epidermal growth factor receptor (EGFR) is elevated in papilloma cells as a result of abnormal receptor recycling (Johnston et al., 1999; Vambutas et al., 1993). Consequently, MAP kinase is constitutively activated and PI3 kinase activity is enhanced in papilloma cells. In contrast, activity of protein kinase B (PKB or Akt), a known signalling molecule downstream of PI3 kinase, is reduced (Zhang & Steinberg, 2000). This is due, at least in part, to overexpression of the phosphatase and tensin homologue (PTEN), a recently characterized tumour suppressor, in papilloma tissues. Immunohistochemical staining has revealed that the elevated levels of PTEN observed in tissue extracts are indeed a result of overexpression in all cell layers, rather than a reflection of expansion of the suprabasal layer in the lesion (Zhang & Steinberg, 2000).

Point mutations in the PTEN/MMAC1/TEP1 gene (Li & Sun, 1997; Li et al., 1997; Steck et al., 1997) have been linked to Cowden’s disease (Nelen et al., 1997) and have been implied in the development of various tumours (Cairns et al., 1997; Liu et al., 1997; Risinger et al., 1997; Teng et al., 1997). PTEN is a dual protein phosphatase capable of dephosphorylating phosphotyrosine and phosphoserine/phosphothreonine in vitro (Li & Sun, 1997; Li et al., 1997; Myers et al., 1998). PTEN is also a lipid phosphatase that dephosphorylates phosphoinositol 3,4,5-triphosphate [PIns(3,4,5)P3] (Maehama & Dixon, 1998), thereby counteracting the effects elicited by PI3 kinase. The tumour suppression activity of PTEN has been attributed largely to its phospholipid phosphatase activity. A G129E mutation in PTEN that abolished its lipid phosphatase activity while retaining the protein phosphatase activity was identified in a cancer patient (Myers et al., 1998). The ability of PTEN to function as a tumour suppressor is consistent with its ability to
inhibit Akt activation (Cantley & Neel, 1999). Akt was shown recently to activate NF-κB (Ozes et al., 1999; Romashkova & Makarov, 1999) and inhibit Forkhead transcription factors (Brunet et al., 1999). Both were shown to be involved in cell cycle regulation (Guttridge et al., 1999; Kaltzschmidt et al., 1999; Medema et al., 2000; Romashkova & Makarov, 1999) and programmed cell death (Barkett & Gilmore, 1999; Brunet et al., 1999). The mechanism by which PTEN negatively regulates proliferation is not well understood. PTEN expression has been associated with elevated levels of p27kip1 (Cheney et al., 1999) and reduced expression of c-myc (Ghosh et al., 1999). Recent observation that overexpressing PTEN had no effect on cell cycle progression in pRb−/− cells underscored the role of the retinoblastoma protein (pRb) in mediating the function of PTEN (Paramio et al., 1999). The increase in the steady-state level of PTEN in laryngeal papilloma tissues predicts that HPV-infected papilloma cells will grow more slowly than normal laryngeal epithelial cells and that HPV-infected cells will be prone to apoptosis.

STAT3, a member of the signal transducer and activator of transcription (STAT) family, is activated as a result of EGFR activation (Grandis et al., 1998; Zhong et al., 1994). Furthermore, STAT3 has recently been classified as a proto-oncogene (Bromberg et al., 1999) and has been shown to play a pivotal role in EGF-induced proliferation in head and neck squamous carcinoma cells (Grandis et al., 1998). Activation of STAT3 is induced by activation of receptor tyrosine kinase, for example EGFR, or by non-receptor tyrosine kinases, for example JAK family kinases, c-Src (Cao et al., 1996; Schaefer et al., 1999) and c-Fes kinase (Nelson et al., 1998). Tyrosine phosphorylation of STAT leads to its nuclear translocation, binding to a specific promoter-proximal element and subsequent transcription activation. An increased STAT3 activation would be expected in HPV-infected papilloma cells due to constitutive activation of the EGFR.

While intensive research has been focused on the lipid phosphatase activity of PTEN and its effect on Akt, little is known about the protein phosphatase activity of PTEN. One obstacle obstructs the identification of its downstream effectors. To date, only the focal adhesion kinase (FAK) (Tamura et al., 1998, 1999) and the adaptor protein Shc (Gu et al., 1999) have been shown to be potential in vivo substrates for PTEN. In contrast with an ever-increasing number of STAT3-activating kinases, there are very few negative regulators of STAT that have been identified. In this study, we report the identification of PTEN as a negative regulator of STAT3 activation in HPV-infected laryngeal papilloma cells.

Methods

**Tissue handling and cell culture.** Surgical discards of normal laryngeal tissues or laryngeal papilloma tissues were derived from patients undergoing laryngeal surgery. The use of human tissues and subsequent culturing were approved by the Institutional Review Board at Long Island Jewish Medical Center. Multiple tissues derived from different patients were used to rule out any aberrant observations limited to a particular patient. Tissues were frozen in liquid nitrogen immediately. Frozen tissues were ground and reduced to a powder by using the Micro-Dismembrator II (Braun). Proteins were extracted by adding lysis buffer, as described below. First-passage laryngeal cells were cultured in KGM medium, as described previously (Johnston et al., 1999). HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% foetal bovine serum.

**Plasmids and antibodies.** pGNC-PTEN and pGNC-G129E mammalian expression constructs (gifts of Michael Myers, Cold Spring Harbor Laboratory) contained, respectively, wild-type and the lipid phosphatase-deficient G129E mutant of PTEN cDNA fused to an N-terminal haemagglutinin (HA) tag (Myers et al., 1999). The fusion proteins were expressed under the control of the cytomegalovirus promiscuous promoter. pTATA-4XM67-Luc and control plasmid pTATA-Luc were gifts of Danile Besser (The Rockefeller University, New York). pHygEGFP was from Clontech. Anti-PTEN polyclonal antibody (N-19), anti-PTEN mouse monoclonal antibody (A2B1) and anti-HA mouse monoclonal antibody (F-7) were purchased from Santa Cruz Biotech. Anti-PTyr(705)STAT3 polyclonal and anti-STAT3(pan) antibodies were purchased from Cell Signaling Technology, Horseradish peroxidase-conjugated antibodies were purchased from Pierce. All detection of phosphorylated STAT3 used the phospho-specific antibody.

**Transfection.** HeLa cells at 60–70% confluence were transfected with various amounts of plasmid DNA using Lipofectamine Plus reagents (Life Technologies) according to the manufacturer’s instructions. Two days after transfection, cells were harvested and lysates were prepared for Western blot analysis. In our hands, this method resulted in 50–70% transfection efficiency for HeLa cells. For EGF stimulation experiments, cells were serum-starved for 12 h before transfection. EGF stimulation was done at a concentration of 100 ng/ml. At indicated times after stimulation, cells were harvested and lysates prepared for Western blot analysis.

**Immunoprecipitation and Western blot analysis.** Cells were lysed on ice with lysis buffer (20 mM HEPES, pH 7.5, 1% Nonidet P-40, 0.4 M NaCl, 10% glycerol, 1 mM DTT) in the presence of Complete protease inhibitor cocktail (Roche Molecular Biochemicals) and phosphatase inhibitors (20 mM 2-glycerophosphate, 1 mM sodium orthovanadate, 30 mM sodium fluoride). After spinning at top speed in a microcentrifuge, the supernatant was transferred to a new Eppendorf tube. The protein concentration of the lysates was determined using Micro BCA reagents (Pierce). For immunoprecipitation, the NaCl concentration of the lysates was adjusted to 150 mM. Antibody (2 μg) was preadsorbed to protein A or protein G beads (Santa Cruz Biotech). The antibody-coated beads were incubated then with 200 μg lysate for 2 h at 4 °C with rocking. The resultant lysates were tested in phosphate assays. For Western blot analysis, 40 μg protein was electrophoresed on an SDS–polyacrylamide gel. Separated polypeptides were transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked with 5% (w/v) non-fat milk and was allowed to react with the primary antibody overnight at 4 °C with rocking. After incubation with the appropriate secondary antibody, the immunoreactive species were detected using SuperSignal West Pico chemiluminescent substrates (Pierce). A commercially available IFN-γ-stimulated HeLa cell lysate was used as a positive control for PTyr(705)STAT3, following the supplier’s instructions (Cell Signaling Technology). Nitrocellulose membranes were stripped to allow detection of additional proteins through the use of a different primary antibody. Thus, membranes were incubated in stripping buffer (62.5 mM Tris–HCl, pHi 6.8, 100 mM β-mercaptoethanol, 2% SDS) at 50 °C for 30 min with occasional shaking. Membranes were washed extensively with Tris-buffered saline (TBS) (10 mM Tris–HCl,
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In vitro phosphatase assay. Lysates were dialysed against phosphatase buffer (50 mM HEPES, pH 7–6, 10 mM MgCl₂, 10 mM DTT) for 4 h with one change of buffer before performing the phosphatase assay. Lysates were incubated in phosphatase buffer at 37 °C for 30 min. Reactions were stopped by adding 5 × SDS gel loading buffer. Dephosphorylation of STAT3 was determined by Western blot analysis.

Luciferase assay. HeLa cells were grown in six-well plates and transfection was performed in triplicate using 1–5 µg pGCN-PTEN or pGCN-G129E or pCDNA3 (Invitrogen), plus 0.5 µg pHygEGFP and 0.5 µg of either pTATA-4XM67-Luc or control pTATA-Luc. Forty-eight hours after transfection, cell lysates were made and luciferase assays were performed using commercial luciferase assay reagents (Promega) and a TD-20 Luminometer (Turner Designs). STAT3-driven luciferase expression was determined by subtracting the background activity obtained in pTATA-Luc transfections after normalization against GFP expression. The final luciferase activity was expressed as a percentage relative to the control pCDNA3 transfection.

Results

The level of phosphotyrosine(705)STAT3 is negatively correlated to PTEN in laryngeal papilloma cells

Previous studies in our laboratory have revealed that MAP kinase and PI3 kinase are activated in laryngeal papillomas (Johnston et al., 1999; Zhang & Steinberg, 2000). Activation of the MAP kinase pathway is likely to be a result of increased levels of EGFR in papilloma tissue. Since STAT3 has been identified as an effector of EGF-induced signalling (Zhong et al., 1994), we reasoned that activation of STAT3 should have been increased in laryngeal papilloma. However, similar to what has been observed for Akt, the level of tyrosine(705)-phosphory-

Fig. 1. The level of PTEN is inversely correlated to that of PTyr(705)STAT3. Lysates were prepared from normal or papilloma tissues. Protein (40 μg) was loaded on each lane and electrophoresed on a 7.5% SDS–polyacrylamide gel. Separated polypeptides were transferred to a nitrocellulose membrane. The membrane was blocked, probed with the appropriate primary antibody and developed as described in Methods. Note that the normal extract was prepared from a pool of two to five normal tissues. A total of six papillomas were studied, with comparable results.

Fig. 2. Papilloma extracts contain a PTyr(705)STAT3 phosphatase activity. Protein (40 μg) from the pooled normal extract (Norm) was incubated with increasing amounts of papilloma extract (Pap) in in vitro phosphatase reactions. Reactions containing only normal or papilloma extract were included for comparison. The relative level of PTyr(705)STAT3 was determined by normalizing the intensity of PTyr(705)STAT3 against total STAT3. Note the increase in total STAT3, due to addition of the papilloma extracts.

Fig. 3. Immunodepletion of PTEN removes PTyr(705)STAT3 phosphatase activity from papilloma extract. Papilloma extracts were immunodepleted with either mouse IgG (control), or N-19 or A2B1 anti-PTEN antibodies. Immunodepleted papilloma extracts were then tested for PTyr(705)STAT3 phosphatase activity in vitro using pooled normal extract as the substrate. A reaction containing only the normal extract was included for comparison. The level of relative PTyr(705)STAT3 was determined by normalizing the intensity of PTyr(705)STAT3 against total STAT3. Note the increase in total STAT3, due to addition of the anti-PTEN antibodies. Data shown are representative results of two separate experiments.
lated STAT3 [PTyr(705)STAT3] was drastically reduced in papilloma tissues in the face of constitutively activated EGFR, compared with the normal control (Fig. 1). In contrast, the steady-state level of STAT3 remained unchanged. The reduced phosphorylation of STAT3 could be inversely correlated to the elevated level of PTEN found in laryngeal papillomas, as previously reported (Zhang & Steinberg, 2000). This observation prompted us to examine further the relationship between PTEN and STAT3 activation.

PTEN participates in dephosphorylating PTyr(705)STAT3

The reduced tyrosine phosphorylation of STAT3 in papilloma tissues could result from either inactivation of an upstream kinase(s) or activation of a downstream phosphatase. To distinguish between these two possibilities, an in vitro phosphatase assay was performed. As illustrated in Fig. 2, reduction in STAT3 tyrosine phosphorylation was observed by adding papilloma tissue extract to a normal cell extract containing activated STAT3 in the phosphatase assay. Furthermore, the remaining STAT3 tyrosine phosphorylation was inversely proportional to the amount of papilloma extract present in the phosphatase reaction. The reduction in phosphorylated STAT3 was greater than would be expected from a simple dilution of the phosphorylated species due to the addition of papilloma extract. This observation indicated the presence of a phosphatase in the papilloma extract that could dephosphorylate PTyr(705)STAT3, at least in vitro. Considering the fact that PTEN is overexpressed in laryngeal papillomas and that PTEN possesses protein phosphatase activity, we hypothesized that PTEN might be an integral component of the PTyr(705)STAT3 phosphatase.

In order to test this hypothesis, a papilloma extract was immunodepleted with a control mouse IgG, with an anti-PTEN polyclonal antibody (N-19) or with an anti-PTEN monoclonal antibody (A2B1). The resultant papilloma extracts were tested for phosphatase activity towards PTyr(705)STAT3 in vitro, as described above. As shown in Fig. 3, whereas control depletion retained PTyr(705)STAT3 phosphatase activity in papilloma extract, extract immunodepleted with either of the PTEN antibodies displayed decreased PTyr(705)STAT3 phosphatase activity. This observation strongly suggested the involvement of PTEN in the phosphatase activity targeting PTyr(705)STAT3.

To further demonstrate an in vivo role for PTEN in negatively regulating PTyr(705)STAT3, we transfected HeLa cells (which have very low levels of endogenous PTEN) with either a haemagglutinin (HA)-tagged wild-type (WT) PTEN cDNA construct or an HA-tagged G129E lipid phosphatase-defective PTEN cDNA construct. Two days after transfection, cells were harvested to determine the level of STAT3 phosphorylation. As indicated in Fig. 4(A), STAT3 phosphory-
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Fig. 5. EGF stimulation of HeLa cells overexpressing PTEN. HeLa cells were either mock-transfected or transfected with pGCN-PTEN (WT) or pGCN-G129E (Mut). Cells were serum-starved for 12 h following transfection and were subsequently stimulated with 100 ng/ml EGF. At indicated times after stimulation, lysates were prepared and levels of PTyr(705)STAT3 and activated EGFR in the lysates were determined by Western blot analysis. The intensity of the activated STAT3 or EGFR was plotted and was expressed as a percentage of the control transfection at zero time of EGF stimulation. Data were averaged from two independent sets of transfections.

PTEN does not inhibit STAT3 kinase activity

The effect of PTEN on STAT3 phosphorylation in vivo could either be due to up-regulation of a phosphatase, as suggested above, or result from inactivation of a STAT3 kinase, or both. To determine whether inactivation of a STAT3 kinase was involved, HeLa cells were serum-starved for 12 h following transfection. Subsequent stimulation with 100 ng/ml EGF resulted in a 2-fold increase in tyrosine-phosphorylated STAT3 in the control (Fig. 5). In cells transfected with either wild-type PTEN or the lipid phosphatase mutant, a more robust stimulation (3–4-fold) was observed. Thus, we concluded that tyrosine phosphorylation of STAT3 is functional in cells overexpressing PTEN, and that the primary effect of PTEN overexpression is a dephosphorylation of STAT3.

Discussion

We report here the identification of PTEN/MMAC1 as a negative regulator of STAT3 activity through mediation of PTyr(705)STAT3 dephosphorylation. Overexpression of PTEN in HPV-infected papilloma cells is due, at least in part, to an increase in expression of the PTEN gene (Zhang & Steinberg, 2000). At present, it is not clear whether this increase results directly from induction by viral protein(s) or indirectly by viral protein(s)-induced alterations of signal transduction. PTEN has attracted much attention since its initial discovery. To date, most studies have focused on the ability of PTEN to dephosphorylate phosphoinositol 3,4,5-triphosphate, which leads to subsequent inactivation of the Akt pathway. It should be noted that PTEN was believed to be a member of the protein tyrosine phosphatase family in the initial study (Li & Sun, 1997). Its phosphotyrosine phosphatase activity was, however, low towards several commonly used substrates (Myers et al., 1997). We have found that recombinant PTEN also showed a mild phosphatase activity against the immunoprecipitated PTyr(705)STAT3 in vitro (data not shown). We propose that the low activity we measured with purified PTEN reflects earlier findings that accessory factors and membrane localization could boost the activity of PTEN (Lee et al., 1999; Wu et al., 2000a, b) and that determination of these factors would be necessary to measure strong STAT3 phosphatase activity in a purified system. However, we have
clearly shown that PTEN-mediated STAT3 dephosphorylation \textit{in vitro} using crude extracts, and overexpression of PTEN in HeLa cells, led to a robust reduction of PTyr(705)STAT3 \textit{in vivo} (Fig. 4). These data strongly suggest that PTEN is an integral component of a STAT3 phosphatase.

STAT3 has recently been classified as a proto-oncogene (Bromberg \textit{et al.}, 1999). STAT3, but not STAT1, is required for EGF-induced growth in head and neck cancer cells (Grandis \textit{et al.}, 1998). Overexpression of PTEN in laryngeal papillomas, with a resultant reduction in activated STAT3, is thus in accord with the reduced rate of proliferation associated with the papilloma cells (Steinberg \textit{et al.}, 1990). The anti-apoptotic activity of STAT3 is also well documented (Fukada \textit{et al.}, 1996; Grandis \textit{et al.}, 2000; Takeda \textit{et al.}, 1998). In this regard, the ability of PTEN to negatively regulate STAT3 activation is consistent with its reported tumour suppressor activity, and with the fact that the HPV 6/11-induced papillomas have a low probability of malignant conversion. It is interesting that HPV 16 (a high-risk HPV)-induced low grade tumour cells display elevated PTEN as well, whereas HPV 16-induced high grade tumour cells contain reduced levels of PTEN (K. Auborn & B.M. Steinberg, unpublished results). This observation implies malignant conversion of HPV-induced lesions upon loss of PTEN. It is also interesting to note that STAT3 was reported to be constitutively active in a plethora of prostate cancer cell lines (Ni \textit{et al.}, 2000), where mutations in PTEN have been frequently detected. It is interesting to note that overexpression of the lipid phosphatase-deficient form of PTEN resulted in greater reduction of PTyr(705)STAT3 (Fig. 4). It remains to be seen whether eliminating PTEN’s lipid phosphatase activity augments its protein phosphatase activity \textit{in vitro}, as our data implied. It is also possible that additional regulatory factors, either activators or repressors of PTEN, are involved in the augmentation of activity. In this case, activation of Akt might activate the activators or inactivate the repressors. Experiments to clarify these possibilities are under way.

It is surprising to learn that both Akt and STAT3 are negatively regulated in HPV-infected laryngeal papilloma cells. Aside from their oncogenic potential, both Akt and STAT3 are known anti-apoptotic regulators. Inhibition of activation of Akt and STAT3 in HPV-infected papilloma cells implies a skew of survival/death signal transduction to the apoptotic side, and one must wonder what advantage would accrue to the virus by inducing such an effect in the target cells. To this end, the observed increase in PTEN levels might be interpreted as a host defence mechanism to reduce cell proliferation and induce cell death in virus-infected cells. However, HPV-infected laryngeal papilloma cells do not die.

Another possible explanation involves the fact that HPV productively replicates in differentiated cells, but not in basal proliferating cells (Flores & Lambert, 1997; Stoler \textit{et al.}, 1989). To this end, induction of a cell cycle-arresting molecule may promote differentiation and ultimately viral replication.

Fig. 6 illustrates our model for the dual activity of PTEN in controlling two proliferative/survival pathways activated by EGF. STAT3 is tyrosine-phosphorylated through activation of EGFR. This may involve c-Src and/or Jak. PTEN that has been recruited to the plasma membrane is responsible for inactivating tyrosine-phosphorylated STAT3, thereby inhibiting Akt activation. PTEN also dephosphorylates PTyr(705)STAT3, thereby negatively regulating STAT3 activation. The model proposes coordinated regulation of both Akt and STAT3 pathways by PTEN.

We hypothesize that PTEN belongs to a membrane-bound signallingome that contains the components of both the PI3 kinase signalling pathway and the STAT3 signalling pathway. PTEN dephosphorylates PIns(3,4,5)P3, thereby inhibiting Akt activation. PTEN also dephosphorylates PTyr(705)STAT3, thereby negatively regulating STAT3 activation. The model proposes coordinated regulation of both Akt and STAT3 pathways by PTEN.
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