Stability of Phosphoprotein as a Biological Marker of Tumor Signaling

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Abstract

Purpose: The purpose of the study was to evaluate the stability of phosphoprotein as a marker of signaling activity in human tumors using clinical samples and xenografts.

Experimental Design: The expression of phospho-Ser473 Akt (p-Akt) was assessed by immunohistochemistry in paraffin-embedded samples from patients enrolled in a Southwest Oncology Group clinical trial of gastroesophageal junction tumors and by immunohistochemistry and Western blotting in human colon tumor xenografts at various times after removal from the animal.

Results: Clinical samples had evaluable p-Akt staining only when obtained as biopsies (9 of 13) and no staining was observed in tumors obtained as surgically resected samples (0 of 15). In HT-29 colon cancer xenografts, p-Akt staining was present in fresh sample but not in tissue that had been allowed to stand for 30 minutes at room temperature. Western blotting of HT-29 tumor xenografts at room temperature showed a slow decrease in total Akt with a half-life of 180 minutes and a rapid decrease in p-Akt with a half-life of 20 minutes.

Conclusions: Caution should be used when using phosphoprotein levels in human tumor specimens to measure intrinsic signaling activity or drug effects because of the potential for rapid dephosphorylation. Rapid processing of biopsies is essential and postoperative surgical samples may be of limited value because of the time to fixation.

Phosphorylation of proteins either on specific tyrosine or serine residues is a posttranslational event modulating the activity or subcellular localization of many key signaling molecules in the cell (1). As such, protein phosphorylation is frequently used as an indicator of signaling activity in the cell. To be useful as a signaling event, protein phosphorylation has to be transient and the phosphorylation is a balance between the rate of phosphorylation by specific kinases and dephosphorylation by phosphatases that may be specific or less specific (2).

Akt is a serine/threonine kinase that is activated by phosphoinositide (PtdIns)-3-kinases (3). p110 PtdIns-3-kinase is an oncogenic protein that can cause cellular transformation (4). Constitutive activation of PtdIns-3-kinase occurs in colon cancer (5), human small cell lung cancer (6), and in ~40% of human ovarian, head and neck, urinary tract, and cervical cancers (7). The major mechanism for the oncogenic activity of PtdIns-3-kinase is by the downstream activation of Akt (protein kinase B) to promote cell survival (8, 9). There are three Akts and all bind through an NH2-terminal pleckstrin homology domain to membrane PtdIns(3,4,5)P3, resulting in their activation by phosphorylation (on Thr308 and Ser473 in Akt1) by membrane-associated PDK1 (10, 11) and another kinase whose identity is not yet clear (12, 13). Phosphorylated Akt detaches from the plasma membrane, moving to the cytoplasm and the nucleus (14). It phosphorylates a battery of downstream targets to prevent the expression of death genes or to induce cell survival (15), including the forkhead transcription factor family members (15), the proapoptotic Bcl-2 family member Bad (16), the apoptosis signaling kinase-1, and procaspase-9, the initiator of the caspase cell death cascade (17). Phospho-Akt has become a standard way of assessing the activity of the PtdIns-3-kinase signaling pathway both in cells and in tumors (18). It has recently been reported that phospho-Akt may be a good predictor of response for an already approved agent in non–small cell lung cancer, gefitinib (Iressa, ZD1839; ref. 19). Surprisingly, phospho-Akt seems to be a better predictor of response to gefitinib than epidermal growth factor receptor or phosphorylated epidermal growth factor receptor. With reliable tissue procurement protocols and standard assays for phospho-Akt assessment, it is hoped that targeted clinical trial designs can be utilized for phase II and III drug development; thus reducing the number of patients being treated with tumors not expressing the target results and fewer patients required for accurate responsiveness data (20). Clearly, if phosphoproteins are to be used effectively as markers of signaling activity in tumors, it is critical to have a knowledge of the stability of the protein phosphorylation after the blood supply to the tumor is terminated and before the tumor is fixed to prevent dephosphorylation of the marker.
To address this question, we have evaluated the expression of phospho-Akt in paraffin-embedded samples from patients enrolled in a clinical trial on gastroesophageal junction tumors and human colon cancer xenografts grown in animals.

**Materials and Methods**

**Human tumor xenografts.** HT-29 colon cancer cells were obtained from the American Tissue Type Collection (Rockville, MD) and tested to be Mycoplasma-free using a PCR ELISA kit (Roche Diagnostics, Inc., Indianapolis, IN). The cells were grown in humidified 95% air, 5% CO₂ at 37°C in DMEM supplemented with 10% fetal bovine serum. Approximately 10⁷ HT-29 cells were injected s.c. into the flanks of scid mice and the tumors allowed to grow to 250 to 300 mm³. The animals were killed and tumors rapidly excised and either frozen immediately in liquid N₂ or fixed in 5% buffered formalin, in both cases as 5 mm³ fragments. The remaining tumor was allowed to stand at room temperature for various times before freezing or fixation in the same way.

**Human tumors.** In accordance with local Institutional Review Board regulations and after obtaining patient informed consent, small diagnostic biopsies or surgically resected specimens were obtained from patients with unresectable or metastatic adenocarcinoma of the esophagus or gastroesophageal junction as part of a Southwest Oncology Group Trial 0127. The samples were typical tumor bank material obtained by multiple participating institutions collected between September 2002 and May 2004. The biopsies were fixed immediately in 10% buffered formalin. The resected specimens were processed for routine pathologic examination with an indeterminate amount of time before fixation in 10% buffered formalin.

**Immunostaining for phospho-Ser⁴⁷³-Akt.** Formalin-fixed, paraffin-embedded tissue sections of 4 μm thickness were cut from the blocks, deparaffinized through xylenes and alcohols, blocked in 4% goat serum in PBS for 30 minutes, and treated with a 1:50 dilution of purified anti-phospho-Ser⁴⁷³-Akt rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA) for 15 hours at 4°C. They were then stained on a Ventana ES automated slide stainer using a Basic DAB Detection kit (Ventana Medical Systems, Tucson, AZ).

**Western blotting.** The tumor xenografts were homogenized in 50 mmol/L HEPES buffer (pH 7.5), 50 mmol/L NaCl, 1% Nonidet P40, 0.25% sodium deoxycholate, 0.2 mol/L sodium fluoride, 0.2 mol/L sodium PPi, and 0.2 mmol/L sodium vanadate. Fifty micrograms of total cell lysate protein were boiled for 5 minutes, loaded on a 12% acrylamide/bisacrylamide gel, and separated by electrophoresis at 160 V for 40 minutes. Proteins were electrophoretically transferred to a polyvinylidene fluoride membranes, preincubated with a blocking buffer of 137 mmol/L NaCl, 2.7 mmol/L KCl, 897 mmol/L CaCl₂, 491 mmol/L MgCl₂, 3.4 mmol/L Na₃HPO₄, 593 mmol/L KH₂PO₄, and 5% bovine serum albumin, and incubated overnight with rabbit purified anti-phospho-Ser⁴⁷³-Akt antibody or anti-Akt antibody (Cell Signaling Technology). Detection used donkey anti-rabbit IgG peroxidase coupled secondary antibody and the Renaissance chemiluminescence system on Kodak X-Omat Blue XB films. Bands were quantified using Eagle Eye software (Stratagene Corp., La Jolla, CA). Tumor Akt activity was expressed as the ratio of phospho-Ser⁴⁷³-Akt to total Akt.

![Fig. 1. Phospho-AKT in human gastroesophageal tumors and HT-29 colon cancer xenografts measured by immunohistochemical staining. Staining used phospho-Ser⁴⁷³-Akt antibody. A, patient tumor samples. 1 and 2 are two surgically resected specimens and 3 and 4 are two biopsy specimens. B, HT-29 human tumor xenografts excised from scid mice and kept at room temperature for the times shown. Small pieces were fixed in 5% formalin for immunohistochemistry. Each section also includes in the upper right-hand quadrant an on-slide control of HT-29 colon cancer cells stained for phospho-Ser⁴⁷³-Akt.](image1)

![Fig. 2. Stability of phospho-Akt in HT-29 human colon cancer xenografts. HT-29 human tumor xenografts were excised from scid mice and kept at room temperature for the times shown. Small pieces were then rapidly frozen in liquid N₂ for Western blotting. A, phospho-Ser⁴⁷³-Akt and total Akt measured by Western blotting. B, time course of the loss of total Akt (●) and phospho-Ser⁴⁷³-Akt (○) relative to total Akt.](image2)
Results

Immunohistochemical staining in human tumor samples. Immunohistochemical staining for phospho-Ser\(^{473}\)-Akt in human adenocarcinoma tumor samples gave variable results (Fig. 1A). Evaluative staining was seen only in tumor samples that were obtained as biopsies (9 of 13), whereas no staining was observed in tumors obtained as surgically resected samples (0 of 15). Therefore, we investigated the stability of phospho-Ser\(^{473}\)-Akt in HT-29 human colon cancer xenograft samples fixed immediately upon excision from the scid mouse or allowed to stand at room temperature before fixation (Fig. 1B). Whereas phospho-Ser\(^{473}\)-Akt staining was apparent in the fresh sample, there was no staining in tissue that had been allowed to stand for 30 to 60 minutes.

Western blotting in tumor xenografts. To distinguish between a loss of phospho-Ser\(^{473}\)-Akt and total Akt and to obtain a more accurate time course, we carried out Western blotting studies using HT-29 human tumor xenografts that were allowed to stand at room temperature before rapid freezing in liquid N\(_2\) (Fig. 2). There was a slow decrease in total Akt with a half-life of 180 minutes and a rapid decrease in phospho-Ser\(^{473}\)-Akt with a half-life of 20 minutes.

Collecting human surgical samples. With the information that there was rapid loss of phospho-Ser\(^{473}\)-Akt staining, we attempted the rapid collection of a surgically resected human colon cancer specimen. The most rapid collection we could achieve between clamping of the blood supply by the surgeon and release of the tissue by the pathologist was 20 minutes. There was no detectable phospho-Ser\(^{473}\)-Akt staining in the samples. The fact that the tumor was at body temperature for a good part of this time may have contributed to the more rapid loss of phospho-Ser\(^{473}\)-Akt staining than in the tumor xenograft samples at room temperature.

Discussion

Phosphorylated proteins are frequently used to assess the activity of intracellular signaling pathways and the effects of cancer drugs to inhibit these pathways (2). When this is done in human tumor samples, the speed with which the tumor specimen can be fixed for immunohistochemical staining or Western blotting can have a major effect on the strength of the signal that is seen. We encountered a striking difference in the phospho-Ser\(^{473}\)-Akt immunostaining between biopsies where staining was strong and postoperative surgical samples where staining was absent. We found that the half-life of phospho-Ser\(^{473}\)-Akt measured by Western blotting in human HT-29 human colon tumor xenografts at room temperature was 20 minutes, whereas total Akt was lost with a half-life of 180 minutes. We had seen a clear correlation between phospho-Ser-Akt staining and whether human adenocarcinoma samples were obtained as biopsies or as surgical specimens, with detection only being seen in biopsies. Biopsies are taken fresh from tumors and likely to be fixed relatively rapidly. Surgical tumor specimens have their blood supply tied and there may be many minutes in the body at 37 °C before the tumor is removed and fixed; in our hands, this took at least 20 minutes. Presumed dephosphorylation at 37 °C is even faster than at room temperature, although this was not measured directly. The stability of phosphoproteins may depend on the protein being studied and on the tissue or tumor type. However, rapid processing would seem to be essential for any study using phosphoprotein to measure signaling activity or drug inhibition and postoperative surgical samples may be of limited value for measuring phospho-Akt levels.

References

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