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Short report

Development of human anti-thymidine kinase antibodies

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Introduction

Thymidine kinase (TK) is a key enzyme involved in the nucleoside salvage pathway leading to the intracellular synthesis of thymidylate through the phosphorylation of preformed thymidine nucleosides. It has previously been shown that cellular TK levels are regulated by intracellular levels of dTTP.[1] A recent study from our laboratory has shown that exposure to camptothecin analogs, such as 9-aminocamptothecin, results in the inhibition of TK through an indirect mechanism not associated with changes in intracellular dTTP pools.[2]

To enable further investigation of this inhibitory mechanism and to provide a reagent useful for other investigations of TK, we have developed anti-TK antibodies in rabbits using peptides of TK. TK is an important determinant of cellular sensitivity to fluoropyrimidines and to several new antifolate inhibitors of thymidylate synthase.[1] Thus, the availability of antibodies against TK would facilitate investigations of the role of this enzyme as a potential predictor of responsiveness to these commonly used chemotherapeutic agents.

Materials and methods

Production of antibody

To generate the antibodies, three 15-amino-acid peptides were synthesized and conjugated to the diptheria toxoid protein. The choice for the three peptides was based on the combination of two different prediction algorithms designed to determine the best sequence for antibody production, Preditop Alogorithm (Chiron Mimotopes, Raleigh, NC) and the Antigenic Index Plot in the MacVector Program (MacVector, Oxford Molecular Group). The three sequences chosen were ^{56}AKDTRYSSSFCTHDR^{70} (rabbit 1), ^{191}KASGQPAGPDNKENC^{205} (rabbit 2) and ^{7}PTVLPGSPSKTRGQI^{21} (rabbit 3). The antibodies were prepared by the Laboratory of Animal Medicine at the NCI–Frederick Cancer Research and Development Center (Frederick, MD). Antibodies were generated by injecting 1 mg of the conjugate in PBS with complete Freund's adjuvant intramuscularly in rabbits. Sera were bled from each of the three rabbits weekly for testing beginning on day 7 after the first injection.

Cell culture

Human HCT116 colon cancer cells were maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) and supplemented with 10% fetal bovine serum (Life Technologies). The other colon cancer cell lines used (HCT115, H630, H630R10, HCT8 and HT29) were also maintained in RPMI 1640 medium with 10% fetal bovine serum. The cell lines were kept incubated at 37°C in a 5% CO_2 atmosphere.

Cellular extract and protein preparation

The reactivity of each of the bleeds was tested against two different sources of TK protein. Total cell lysates were harvested from the human HCT116 colon cancer cells according to published methods[3] and were used as one TK source as this line is known to contain relatively high levels of TK. TK protein was also translated *in vitro* using the TNT coupled wheat germ extract system (Promega, Madison, WI). The pcDNA3.1/GS plasmid with the human TK cDNA cloned into the plasmid downstream from a T7

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polymerase promoter was used as the template for the reaction. Total cell extracts from the various six colon cell lines used for Western blot analysis and TK catalytic activity comparison were prepared as previously described.[3] The same cell extract for each line was used for both TK level analyses.

Western blot analysis

All protein samples were resolved on a 12.5% SDS–polyacrylamide gel and electroblotted onto a nitrocellulose membrane as previously described.[4] The membrane was incubated with Blotto blocking solution (5% non-fat milk, 10 mM Tris, 0.01% thimerosol) for 1 h and then incubated with the TK antiserum (1:400 dilution in Blotto) for 1 h at room temperature. The blots were washed with 1× PBS/0.02% Tween and incubated with goat anti-rabbit immunoglobin (1- to 600-fold dilution) for 1 h at room temperature. After the washing was complete, the blots were subjected to chemiluminescent substrate (Pierce, Rockford, IL) for signal detection.

Competition for antibody binding

Positive rabbit antiserum was incubated with 1 μg of peptide (same sequence of conjugated peptide used for injection) in 500 μl of Blotto for 1 h at room temperature on a rotational device. The mix was then diluted into 20 ml of Blotto for a final dilution of 1:400 and added to the pre-blocked blot for primary antibody incubation.

TK catalytic activity

The TK catalytic activity was measured in the lysates of the various colon cell lines as previously described.[2] Briefly, the assay was performed in a total reaction volume of 200 μl containing 50 mM Tris, 10 mM ATP, 5 mM $MgCl_2$, 0.1 mM unlabeled thymidine, 10 mM sodium fluoride, 500 000 d.p.m. of [^{3}H]dThd and cell cytosol representing 250 μg of protein. The samples were incubated for 30 min at 37°C. The reaction was quenched by placing samples in boiling water for 1 min. DE81 filters were spotted with a 50 μl aliquot of each sample. The filters were air dried, washed with distilled water and placed in scintillation cocktail for counting in a Beckman liquid scintillation counter.

TK immunocytochemistry

Two cell lines (HCT116 and HCT8) were fixed in 4% paraformaldehyde for 2 h, embedded in paraffin and mounted on poly-L-lysine slides. Following deparaffinization and rehydration in graded alcohol, the slides were incubated in a 95–100°C water bath for 20 min in sodium citrate buffer, pH 6.0. After cooling to room temperature, endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 for 10 min, followed by washes in H_2O and PBS. The slides were then stained using the Vectastain Elite ABC kit (Vector, Burlingame, CA). Following a blocking step in dilute goat serum, TK antibodies were applied at a dilution of 1:2000 for an overnight incubation at 4°C. Subsequent to incubations in biotinylated anti-rabbit secondary antibody, avidin–biotin complex (ABC reagent) and the chromagen DAB (Sigma, St Louis, MO), the slides were counterstained with hematoxylin, cleared and mounted.

Results/discussion

The ability of the antiserum produced by each of the three rabbits to detect TK protein was tested by Western analysis. Antibodies produced by rabbit 2 and

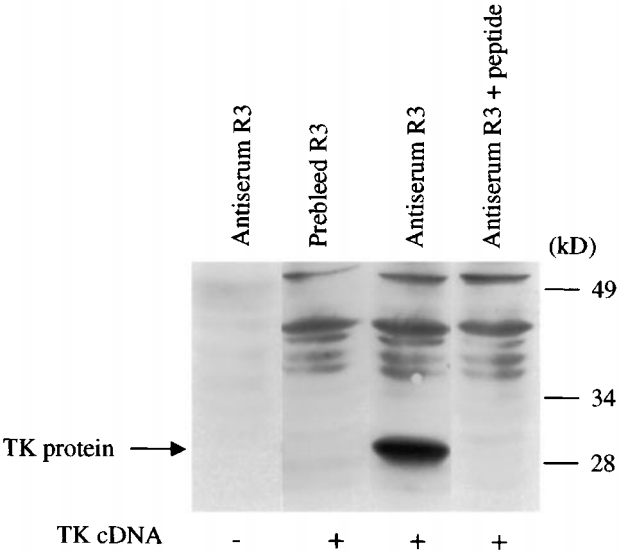

Figure 1. Western immunoblot analysis of *in vitro* translated human TK protein. TK protein was translated using a TNT coupled wheat germ extract system. Translation reactions included either no TK cDNA (lane 1) or 1 μg of TK cDNA (lanes 2–4). The products were resolved on a SDS–polyacrylamide gel and transferred to nitrocellulose as described in the text. Lane 2 was treated with the prebleed serum, and lanes 1 and 3 were incubated with antiserum bled from rabbit 3. In lane 4, the antibody was first preincubated with unconjugated peptide for 1 h at room temperature prior to treating the blot. All lanes were then treated with a secondary anti-rabbit antibody and the protein was detected by chemiluminescence at approximately the 28 kDa marker.

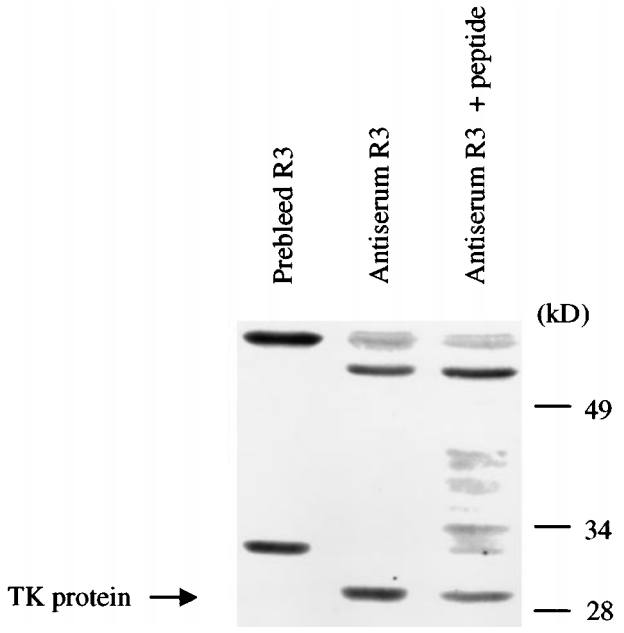

Figure 2. Western immunoblot analysis of cell lysates from the human colon cancer cell line HCT116. Extracts from the cells were prepared as described in the text. Total protein (200 μg) was resolved on a SDS–polyacrylamide gel and transferred to nitrocellulose as described in the text. The protein in lane 1 was treated with the prebleed serum from rabbit 3. Lanes 2 and 3 were both treated with antiserum from rabbit 3; however, the antibody used for lane 4 was first preincubated with unconjugated peptide for 1 h at room temperature prior to treatment of the blot. The TK protein was detected slightly above the 28 kDa marker with an estimated molecular weight of 28 300.

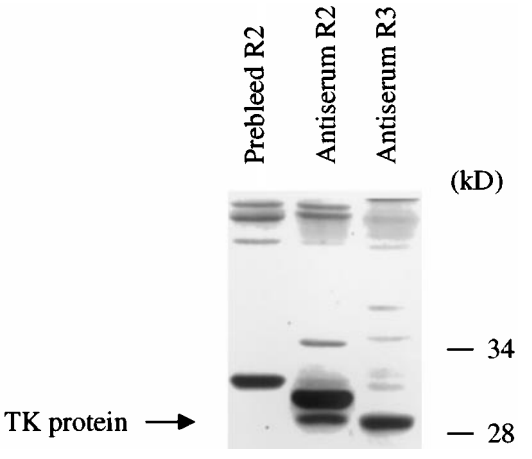

Figure 3. Western immunoblot analysis of cell lysate from the human colon cell line HCT116 comparing antibody isolated from both rabbit 2 and rabbit 3. Lane 1 was treated with the prebleed serum from rabbit 3. Lanes 2 and 3 were treated with antisera (1:400 dilution) isolated from rabbits 2 and 3, respectively. Chemiluminescence was used for detection of the protein.

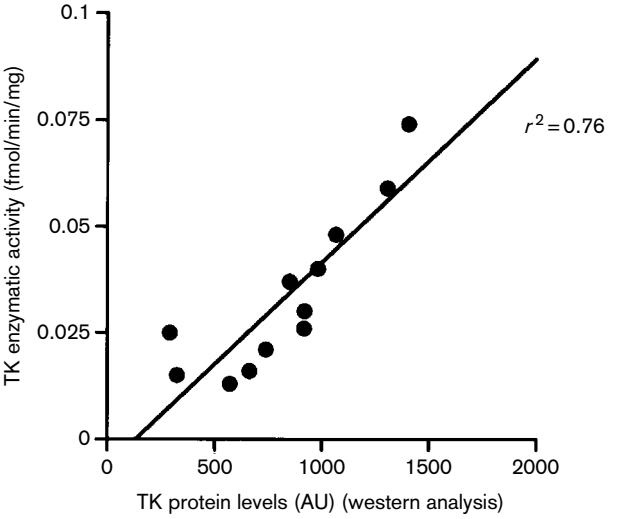

Figure 4. Comparison of TK protein levels detected by Western analysis and TK catalytic activity in six different colon cancer cell lines. The TK protein levels in each cell line were detected by rabbit 3 antibody as described and were quantitated by densitometry. The TK catalytic activity was also determined for each cell line. The protein level (in arbitrary units) and its corresponding enzymatic activity level were plotted for each of the six cell lines. A linear relationship between the two was demonstrated with the indicated correlation coefficient of 0.76.

rabbit 3 but not rabbit 1 were positive for detection of TK protein as illustrated in Figures 1–3. These antibodies were able to detect TK protein from both the HCT116 cell lysate and the *in vitro* translated TK protein. Figure 1 shows the detection of *in vitro* translated TK, indicated by the reactive band migrating slightly above the 28 kDa marker using the antiserum from rabbit 3. This band was not detected using the prebleed serum nor was there a similar band detected in the control sample that did not contain the TK cDNA. Specific binding of this antiserum was confirmed by competition using an unconjugated peptide with the identical sequence as that used to make the antibody. When the antiserum from rabbit 3 was preincubated with the unconjugated peptide, the TK protein was no longer detected as indicated in the last lane of Figure 1.

The antibody produced in rabbit 3 also detected the TK protein in the HCT116 cell lysate as shown in Figure 2. There was no protein detected with the prebleed sample, but a signal was detected with the antiserum, which migrated above the 28 kDa marker with an estimated molecular weight of 28 300. To confirm specificity of the binding of the antibody to the TK protein, unconjugated peptide with the same

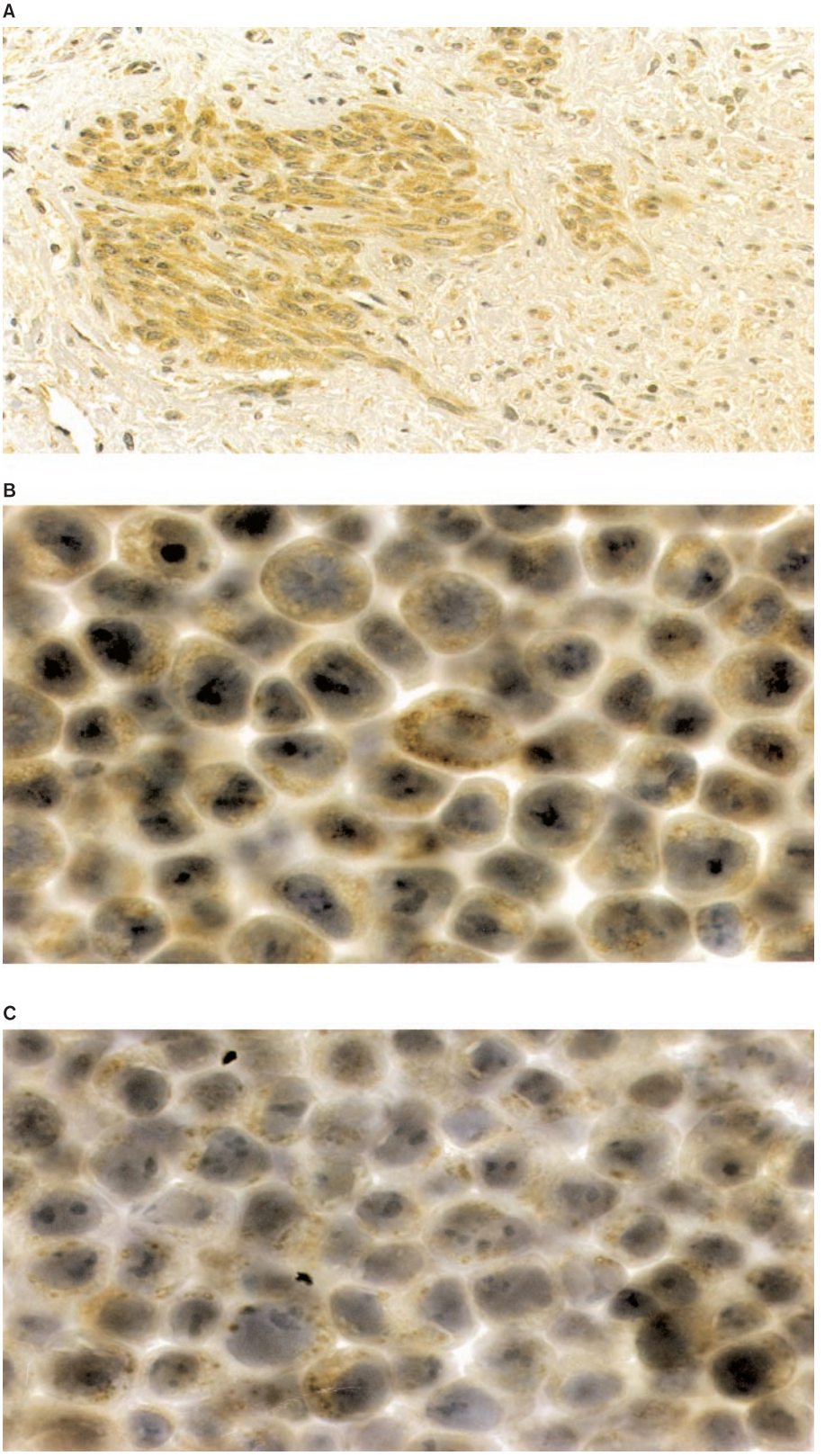

Figure 5. Immunostaining of leiomyoma tissue (A) and two cell lines, including HCT116 (B) and HCT8 (C). Immunostaining was performed using the ABC method as described in Materials and methods.

sequence as that used for rabbit injection was used as a competitor. When the antiserum was preincubated with the unconjugated peptide corresponding to the peptide injected into rabbit 3, the signal is substantially decreased from that seen with the antiserum alone (Figure 2, lane 3).

The antibody produced in rabbit 2 was also found to detect the TK protein in HCT116 cell lysate as shown in Figure 3 (lane 2). Figure 3 demonstrates a comparison of the two positive anti-sera. In addition to TK, the antiserum from rabbit 2 recognized a protein band just above the TK signal at 30.2 kDa.

To further test the specificity of the antisera for TK, six different colon cancer cell lines were chosen to quantitate the levels of TK protein for comparison with TK catalytic activity. As shown in Figure 4, the levels of catalytic activity amongst the six cell lines demonstrate a linear correlation with their respective protein levels as quantitated using Western analysis (r^2=0.76).

The antiserum produced by rabbit 3 was also used to perform immunocytochemical staining in two cell lines that represent the extremes of TK activity amongst the six cell lines in which TK catalytic activity was studied. As shown in Figure 5, the HCT116 cell line which had the highest level of TK catalytic activity (0.074 fmol/min/mg of protein) demonstrated a higher level of staining when compared with the HCT8 (0.021 fmol/min/mg of protein) cell lines, consistent with the differences in catalytic activity measured in these two cell lines. Thus, there was a good correlation between the catalytic activity of TK as measured using the biochemical assay and the intensity of staining. In addition, a tissue section containing leiomyoma cells was stained for TK using the antisera and also demonstrated staining of the leiomyoma cells. In all cases, the TK appeared to be localized primarily in the cytoplasm.

When the peptide sequence of peptides 2 and 3 were compared to TK sequences from other species, a high level of conservation was identified in vertebrate and mammalian sources, but not with the TK sequenced from bacteria or viruses. For the peptide used to immunize rabbit 2, the amino acid conservation ranged from a low of 40 and 47% in the mouse and chicken to 80% in the Chinese hamster. However, the peptide sequence used to immunize rabbit 3, demonstrated an even higher level of conservation amongst each of four species, including chicken (73%), rat (93%), mouse (100%) and Chinese hamster (100%). The high level of homology amongst these species suggests that rabbit 3 antibody may also be useful for detecting and quantitating TK in non-human sources. A search for similar sequences in the available protein databases revealed no matches with either of the sequences used to immunize rabbit 2 or 3, other than TK.

These data illustrate that the rabbit antisera generated by immunization using two peptides of TK recognizes human TK protein from various sources and may be used to accurately quantitate the intracellular levels of TK. These antibodies may have value for predicting sensitivity to nucleotide and antifolate inhibitors of thymidylate synthase and TK, and for further exploratory studies into the mechanism of TK inhibition associated with cellular exposure to camptothecin and camptothecin analogs.

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