Brief report

Anti-Epo receptor antibodies do not predict Epo receptor expression

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Investigators using anti-EpoR antibodies for immunoblotting and immunostaining have reported erythropoietin receptor (EpoR) expression in nonhematopoietic tissues including human tumors. However, these antibodies detected proteins of 66 to 78 kDa, significantly larger than the predicted molecular weight of EpoR (56-57 kDa). We investigated the specificity of these antibodies and showed that they all detected non-EpoR proteins. C-20 detected 3 proteins in tumor cell lines (35, 66, and 100 kDa). Sequences obtained from preparative gels had similarity to the C-20–immunizing peptide. The 66-kDa protein was a heat shock protein (HSP70) to which antibody binding was abrogated in peptide competition experiments. Antibody M-20 readily identified a 59-kDa EpoR protein. However, neither M-20 nor C-20 was suitable for detection of EpoR using immunohistochemical methods. We concluded that these antibodies have limited utility for detecting EpoR. Thus, reports of EpoR expression in tumor cells using these antibodies should be viewed with caution. (Blood. 2006;107:1892-1895)

Introduction

Erythropoietin (Epo) binds to the Epo receptor (EpoR) on the surface of erythroid precursor cells, stimulating their proliferation, survival, and differentiation into mature red cells.[1] Recently, EpoR was reportedly detected in human tumors,[2-12] suggesting that Epo activates EpoR on tumor cells, thereby promoting growth. The anti-EpoR antibodies used to measure EpoR protein in these studies detected 66- to 78-kDa proteins; however, the mature form of human EpoR has a calculated molecular weight of between 56 and 57 kDa. This discrepancy in size suggested that these antibodies cross-reacted with non-EpoR proteins. We therefore examined the specificity of anti-EpoR antibodies used in those studies.

Study design

Cell culture and treatments

The MCF-7, 769-P, SH-SY5Y, HeLa, and Caki-2 cell lines from breast, kidney, brain, cervix, and kidney tumors, respectively, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The megakaryoblastic leukemia cell line, UT-7/Epo,[13] was a gift from Dr Norio Komatsu (Jichi Medical School, Minamikawachi, Japan). The murine leukemia cell line, 32D/Epo, required Epo for growth.[14,15]

Antibodies

We tested 4 commercially available rabbit polyclonal antipeptide anti-EpoR antibodies, including C-20 (sc-695; anti–human EpoR), M-20 (sc-697; anti–mouse EpoR), and H-194 (sc-5624; anti–human EpoR) from Santa Cruz Biotechnology (Santa Cruz, CA), and 07-311 (anti–mouse EpoR) from Upstate Biotech (Waltham, MA). M-20 and 07-311 may also recognize human EpoR. Anti-Flag (M-2) and anti–cyclophilin B were from Sigma-Aldrich (St Louis, MO) and AbCam (Cambridge, MA), respectively.

Gene constructs

Tagged versions of EpoR contained an 8-amino acid N-terminal FLAG sequence attached to full-length EpoR (FLAG-EpoR) or to EpoR with the C-terminal 40 amino acids deleted (FLAG-EpoR Δ40). COS-7 cells were transiently transfected with pcDNA3.1 containing these constructs or with pcDNA3.1 only, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Immunoblotting and peptide-blocking studies

Cells were sonicated in 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 × complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), 100 μg/mL Pefabloc (Roche Applied Science), and 10 μg/mL pepstatin. Reduced and denatured proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes, blocked with 5% milk, and incubated with primary antibody. The secondary antibody was either antimouse or antirabbit conjugated to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ). ECL-Plus (Amersham Biosciences) was used for detection. Imaging was done using Hyperfilm (Amersham Biosciences) and an x-ray developer. For blocking experiments, C-20 was preincubated with peptides and the immunoblots were processed as described. Peptides were synthesized based on the sequence analysis of proteins detected by C-20 and included

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HSP70-2 (QQGRVEILANDQGNRTTPSYVAFTDTER) and HSP70-5 (EIIANDQGNRITPSYVAFTPEGERLIGDAA).

Immunohistochemistry

Sections of fixed cell pellets and whole mouse embryos were blocked with CAS BLOCK (Zymed Laboratories, San Francisco, CA) and incubated with primary antibody. Sections were washed, incubated with biotinylated goat antirabbit antibody (Vector Laboratories, Burlingame, CA), quenched with 3% hydrogen peroxide, and incubated with avidin-biotin complex conjugated to HRP (Vector Laboratories). Reaction sites were visualized with DAB (Dako, Carpinteria, CA) and counterstained with hematoxylin. Images were acquired using a Nikon Eclipse E600 microscope with a Nikon DXM1200F digital camera attached through a Nikon Plan Apo 2 ×/0.1 numeric aperture lens, using ACT-1 software for DXM1200 and DXM1200F (all from Nikon, Melville, NY). Composite images were assembled using Adobe Photoshop version 5.5 (Adobe Systems, San Jose, CA).

Sequence analysis of proteins bound by C-20

Proteins immunoprecipitated by C-20 from UT-7/Epo cells were separated by SDS-PAGE, excised from the gel, reduced and carboxyamidomethylated, and subjected to overnight tryptic in-gel digestion as described.[16] Peptides were separated by high-performance liquid chromatography (HPLC) and analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) on a Thermo Finnigan LCQ DECA XP (San Jose, CA). Peptide sequences determined from MS/MS fragment ion spectra were searched against a nonredundant database to identify full-length proteins. Protein sequences that had at least a 5-amino acid identity to the C-20–immunizing peptide (C-20p) were analyzed further.

Results and discussion

We performed a literature search on EpoR expression in tumors and identified 4 commercially available anti-EpoR antibodies, H-194, C-20, M-20, and 07-311. Each antibody detected recombinant full-length FLAG-EpoR (Figure 1), but the observed size of this protein was 59 kDa, significantly less than the size of EpoR suggested by investigators and the manufacturers of the antibodies (64-72 kDa).[6-8,10]

Each antibody detected additional proteins in tumor cells, ranging from 3 for C-20 to over 20 for H-194 (Figure 1). C-20 detected 3 dominant proteins of approximately 35, 66, and 100 kDa in all tumor cell lines and detected a protein similar in size to FLAG-EpoR in UT-7/Epo cells (Figure 1F). This is consistent with a report that C-20 detects a 66-kDa protein in tumor cell lines.[6,10] However, using a variety of methods, we showed that the 35-, 66-, and 100-kDa proteins were not EpoR nor derived from EpoR; knockdown of EpoR expression in SH-SY5Y cells did not prevent C-20 from detecting these proteins (Figure S1, available on the *Blood* website; see the Supplemental Figures link at the top of the online article), and C-20 immunoprecipitated the 59-kDa protein from UT-7/Epo cells, but not the 66-kDa protein. The immunocomplexed 59-kDa protein was detected by 07-311 and M-20 (Figure S2), suggesting it was EpoR.

Sequence analysis confirmed EpoR peptide sequences in the 59-kDa protein band but not in the 66-kDa protein band. One third

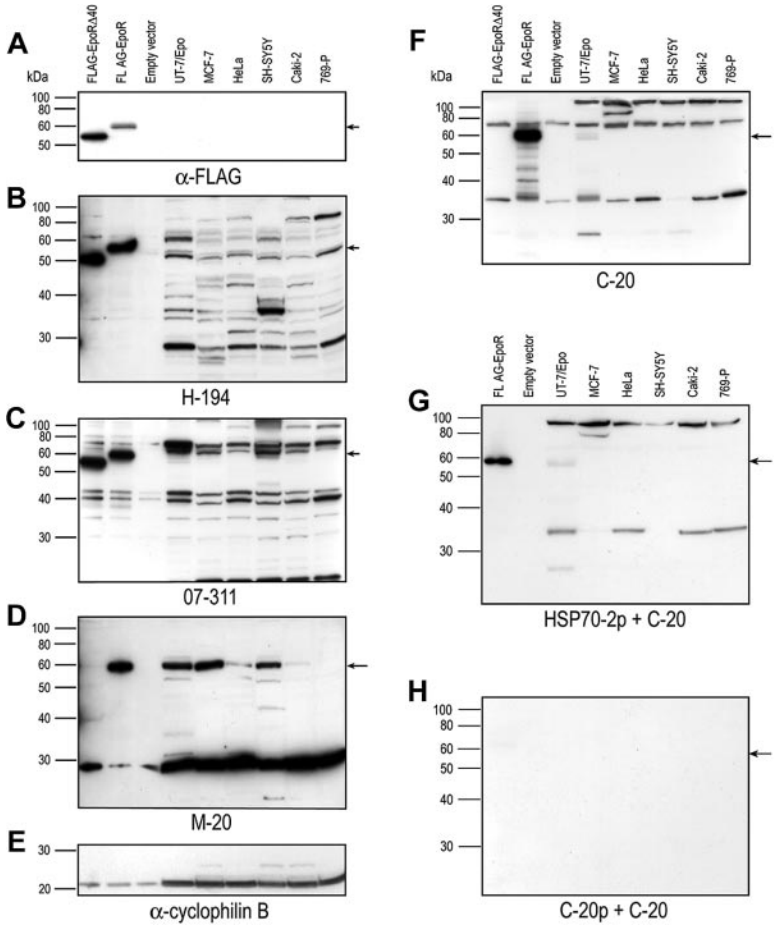

Figure 1. Detection of EpoR with anti-EpoR antibodies. Protein extracts were prepared from tumor cell lines and COS-7 cells expressing FLAG-EpoR and subjected to Western immunoblotting. Each antibody was used to probe blots that had been processed simultaneously: (A) Anti-FLAG (M-2) antibody (1.8 μg/mL); (B) H-194 (0.03 μg/mL); (C) 07-311 (0.4 μg/mL); (D) M-20 (0.2 μg/mL); and (E) blot D was stripped and then reprobed with anti–cyclophilin B antibody (0.25 μg/mL) as a loading control; (F) C-20 (1.32 μg/mL); (G) C-20 preincubated with a 50-fold mass excess of HSP70-2 peptide (note absence of 66-kDa band); (H) C-20 preincubated with 10-fold mass excess of C-20p. The arrows indicate the position of the 59-kDa proteins. The positions of the molecular weight markers are illustrated on the left (kDa). FLAG-EpoR Δ40 protein was detected by the H-194 and 07-311 antibodies that recognize the extracellular domain and the N-terminal 15 amino acids, respectively, of EpoR.

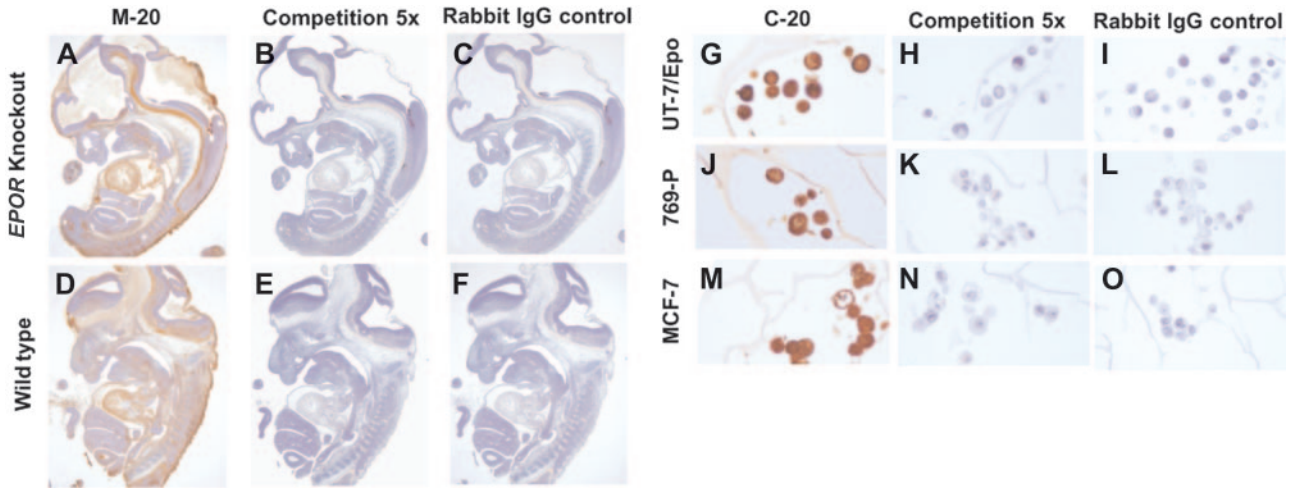

Figure 2. Lack of specificity of M-20 and C-20 antibodies when used for immunohistochemistry. To obtain *EPOR*-knockout ($EPOR^{-/-}$) mouse embryos, timed matings were performed with heterozygous $EPOR^{+/-}$ mice.[21] $EPOR^{+/+}$ and $EPOR^{-/-}$ embryos (determined by yolk sac DNA polymerase chain reaction genotyping[21]) were recovered at day 12.5 for histology of the complete embryo and at day 13.5 for liver tissue. $EPOR^{-/-}$ embryos and $EPOR^{+/+}$ embryos from wild-type littermates stained with M-20 (A,D). $EPOR^{-/-}$ and $EPOR^{+/+}$ embryos stained with M-20 preincubated with a 5-fold excess of M-20 peptide (B,E). Rabbit IgG control antibody (C,F). UT-7/Epo, 769-P, and MCF-7 cell lines stained with C-20 (G,J,M). C-20 preincubated with a 5-fold excess of C-20p (H,K,N). Rabbit IgG control antibody (I,L,O).

of the peptides identified in the 66-kDa band were from HSPs including HSP70-2 and HSP70-5. These proteins contained regions of similarity to the immunizing peptide (C-20p), suggesting that C-20 cross-reacted to these regions. This was confirmed in peptide-blocking experiments, where the HSP70-2 (Figure 1G) and HSP70-5 (data not shown) peptides specifically inhibited C-20 binding to the 66-kDa protein. In contrast, C-20p blocked binding of C-20 to all proteins on the blot (Figure 1H). HSP70 was elevated in many human carcinomas[17,18] and increased with hypoxia.[19,20] Thus, reports that EpoR was overexpressed in tumor cells may be attributed to detection of HSP70 by C-20.

In Western analysis, M-20 detected a 59-kDa protein in all human tumor cell lines except 769-P (Figure 1D). M-20 did not detect the 59-kDa protein in fetal liver cells from $EPOR^{-/-}$ knockout mice (Figure S3), and expression was reduced in SH-SY5Y cells that expressed *EPOR* shRNA (Figure S2). These results confirmed that M-20 can be used for detection of EpoR in Western blots and that the 59-kDa protein is EpoR.

However, using M-20, there was no difference in staining of tissue from *EPOR*-knockout ($EPOR^{-/-}$) and wild-type ($EPOR^{+/+}$) mice (Figure 2A,D). Thus, although M-20 was suitable for detection of EpoR in immunoblots, it was not suitable for immunohistochemistry. When M-20 was preincubated with a 5-fold mass excess of M-20–immunizing peptide (M-20p), staining was absent in both wild-type and knockout tissues (Figure 2B,E), suggesting the M-20 staining was due to cross-reactivity to non-EpoR proteins. As expected, no staining was observed with IgG control antibodies (Figure 2C,F).

C-20 has also been used extensively to detect EpoR in tumor cells using immunohistochemistry methods, and investigators have reported that EpoR is overexpressed in tumor cells.[2-6,10-12] Using C-20, we observed similar intensities of staining in UT-7/Epo, MCF-7, and 769-P cells that contained high, moderate, and undetectable levels of EpoR, respectively, indicating that C-20 has insufficient specificity to determine EpoR expression. Cell staining was eliminated by preincubating C-20 with C-20p (Figure 2H,K,N), and no staining was observed with IgG control antibody (Figure 2I,L,O). Therefore, these controls do not validate the use of C-20 for immunohistochemistry.

In conclusion, this study shows that only M-20 is suitable for detection of the 59-kDa EpoR by immunoblotting, and none of the antibodies are suitable for detection of EpoR by immunohistochemistry. Thus, studies alleging EpoR expression using these antibodies should be interpreted with caution.

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References

1. Jelkmann W. Molecular biology of erythropoietin. Intern Med. 2004;43:649-659.
2. Arcasoy MO, Amin K, Chou SC, Haroon ZA, Varia M, Raleigh JA. Erythropoietin and erythropoietin receptor expression in head and neck cancer: relationship to tumor hypoxia. Clin Cancer Res. 2005;11:20-27.
3. Acs G, Zhang PJ, Rebbeck TR, Acs P, Verma A. Immunohistochemical expression of erythropoietin and erythropoietin receptor in breast carcinoma. Cancer. 2002;95:969-981.
4. Arcasoy MO, Amin K, Vollmer RT, Jiang X, Demark-Wahnefried W, Haroon ZA. Erythropoietin and erythropoietin receptor expression in human prostate cancer. Mod Pathol. 2005;18:421-430.
5. Acs G, Xu X, Chu C, Acs P, Verma A. Prognostic significance of erythropoietin expression in human endometrial carcinoma. Cancer. 2004;100:2376-2386.
6. Acs G, Acs P, Beckwith SM, et al. Erythropoietin and erythropoietin receptor expression in human cancer. Cancer Res. 2001;61:3561-3565.
7. Westenfelder C, Baranowski RL. Erythropoietin stimulates proliferation of human renal carcinoma cells. Kidney Int. 2000;58:647-657.
8. Selzer E, Wacheck V, Kodym R, et al. Erythropoietin receptor expression in human melanoma cells. Melanoma Res. 2000;10:421-426.
9. Ribatti D, Marzullo A, Nico B, Crivellato E, Ria R, Vacca A. Erythropoietin as an angiogenic factor in gastric carcinoma. Histopathology. 2003;42:246-250.
10. Acs G, Zhang PJ, McGrath CM, et al. Hypoxia-inducible erythropoietin signaling in squamous dysplasia and squamous cell carcinoma of the uterine cervix and its potential role in cervical carcinogenesis and tumor progression. Am J Pathol. 2003;162:1789-1806.
11. Dagnon K, Pacary E, Commo F, et al. Expression

of erythropoietin and erythropoietin receptor in non-small cell lung carcinomas. Clin Cancer Res. 2005;11:993-999.

12. Eccles TG, Patel A, Verma A, et al. Erythropoietin and the erythropoietin receptor are expressed by papillary thyroid carcinoma from children and adolescents. Expression of erythropoietin receptor might be a favorable prognostic indicator. Ann Clin Lab Sci. 2003;33:411-422.
13. Komatsu N, Yamamoto M, Fujita H, et al. Establishment and characterization of an erythropoietin-dependent subline, UT-7/Epo, derived from human leukemia cell line, UT-7. Blood. 1993;82:456-464.
14. Migliaccio AR, Migliaccio G, D'Andrea A, et al. Response to erythropoietin in erythroid subclones of the factor-dependent cell line 32D is determined by translocation of the erythropoietin receptor to the cell surface. Proc Natl Acad Sci U S A. 1991;88:11086-11090.
15. Greenberger JS, Sakakeeny MA, Humphries RK, Eaves CJ, Eckner RJ. Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. Proc Natl Acad Sci U S A. 1983;80:2931-2935.
16. UCSF Mass Spectrometry Facility. UCSF In-Gel Digest Procedure. http://donatello.ucsf.edu/in-gel.html. Accessed December 15, 2005.
17. Hwang TS, Han HS, Choi HK, et al. Differential, stage-dependent expression of Hsp70, Hsp110 and Bcl-2 in colorectal cancer. J Gastroenterol Hepatol. 2003;18:690-700.
18. Leist M, Jaattela M. Burning up TNF toxicity for cancer therapy. Nat Med. 2002;8:667-668.
19. Giffard RG, Xu L, Zhao H, et al. Chaperones, protein aggregation, and brain protection from hypoxic/ischemic injury. J Exp Biol. 2004;207:3213-3220.
20. Sharp FR, Kinouchi H, Koistinaho J, Chan PH, Sagar SM. HSP70 heat shock gene regulation during ischemia. Stroke. 1993;24:I72-I75.
21. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell. 1995;83:59-67.
22. Sinclair A, Busse L, Rogers N, Sarosi I, Van G, Elliott S. Is EPO receptor expressed in human tumor cells? [abstract]. Blood. 2004;104:17b. Abstract 3723.
23. Busse L, Sinclair A, Rogers N, Sarosi I, Van G, Elliott S. Is EPO receptor over-expressed in human tumor cells? [poster]. Proc Amer Assoc Cancer Res. 2005;46. Abstract 4562.
24. Elliott S, Bass MB, Spahr C, et al. Validation of methodologies used to detect erythropoietin receptor expression in tumor cells [poster]. Pharmacotherapy. 2005;25:1533.