Response:

Anti-Epo receptor antibodies do not predict Epo receptor expression

In this issue, Verdier et al comment on our recent publication, concluding that some anti-EpoR antibodies have limited utility for detecting EpoR expression.1 We reported that the C-20 anti-EpoR antibody from Santa Cruz Biotechnology (Santa Cruz, CA) detected 5 protein bands in UT-7/Epo cells including 59-kDa and 66-kDa proteins. Using both direct and indirect methods, we showed that the 59-kDa protein, not the 66-kDa protein, is EpoR: (1) the 59-kDa protein migrated similarly to recombinant, FLAG-tagged, full-length EpoR; (2) the 59-kDa protein levels decreased following EPOR shRNA treatment of cells, whereas 66-kDa protein levels remained unchanged; (3) the 59-kDa protein was absent in EPOR-knockout fetal liver but present in wild-type fetal liver; (4) C-20 immunoprecipitated the 59-kDa protein, not the 66-kDa protein, from UT-7/Epo cells (the immunocomplexed 59-kDa protein was detected by 2 other anti-EpoR antibodies, 07-311 and M-20); (5) the 59-kDa, not the 66-kDa, protein bands contained EpoR peptide sequences; and (6) peptides derived from heat-shock proteins specifically inhibited C-20 binding to the 66-kDa protein.

Agreeing that many antibodies are unsuitable for detecting EpoR, the authors also do not challenge our conclusion that C-20 should not be used for immunohistochemistry. However, they disagree about the utility of C-20 for detecting EpoR proteins in Western blots and that the apparent molecular mass of EpoR is approximately 59 kDa, not 66/78 kDa as described in the product information sheet provided by Santa Cruz.

Verdier et al claimed that 3 protein bands larger than 59 kDa (64, 67.6, and 69.5 kDa) detected in UT-7 cells by C-20 were different forms of EpoR, based on indirect methods. First, they showed that the 64-kDa protein was not precipitated by C-20 in EpoR-negative Mo7E cells, although an approximately 68-kDa protein was detected in the same cells. Second, they showed that the levels of these proteins were altered following treatment with cycloheximide or stimulation with Epo. However, neither agent necessarily selectively alters EpoR levels. Third, they used an in-house anti-EpoR antibody (C-236), biotinylated Epo/streptavidin, and an anti-Epo antibody to immunoprecipitate the putative EpoR proteins, but each yielded different protein patterns. For example, 2 proteins were immunoprecipitated by C-236 and only one by biotinylated Epo/streptavidin or the anti-Epo antibody. Furthermore, when probed with a second batch of C-20 (lot B2105), the negative control anti-GST antibody appeared to precipitate the same 2 putative EpoR proteins as C-236. Finally, they showed a predominant 64-kDa protein in BaF3 cells transfected with EPOR; expression of the larger 67.6- and 69.5-kDa proteins did not increase in this experiment. Based on their results, we do not believe that Verdier et al have adequately demonstrated that the larger proteins detected by C-20 are EpoR. A possible explanation for the discrepancies between the 2 studies is that the 64-kDa protein detected by Verdier et al is the same as the 59-kDa EpoR protein we report, whereas the larger proteins are non-EpoR cross-reacting proteins. The reported size difference (59 vs 64 kDa) may be a consequence of different conditions used to resolve the proteins or the use of different marker proteins to estimate size.

Steve Elliott, Angus M. Sinclair, and C. Glenn Begley

Correspondence: Steve Elliott, Amgen Inc, One Amgen Center Dr, Thousand Oaks, CA 91320; e-mail: selliott@amgen.com.

The authors are employed by Amgen Inc, a manufacturer and distributor of erythropoietic-stimulating proteins.

Reference


To the editor:

Erythropoietin receptors on cancer cells: exciting perspectives, difficult to appreciate

In a recent article, Elliott et al1 question the specificity of commercially available antibodies against the erythropoietin receptor (EpoR) and thus the validity of an increasing number of studies showing expression of functional EpoR in many nonhematopoietic tissues including most solid cancers.2-6 It is well known that antibody qualities are quite variable. The effort of Elliott et al1 to thoroughly test the available EpoR antibodies should therefore be appreciated, because the authors could eventually have contributed to this field of research. However, a closer look at their study reveals severe methodological issues. First, Elliott et al1 used an expression construct for EpoR that has not been verified in this study. Unfortunately, the detected band was not subjected to liquid chromatography coupled to mass spectrometry (LC-MS), functional integrity of the expressed protein was not demonstrated, and it is therefore unclear if the expression plasmid codes for a functional human EpoR. Second, EpoR undergoes posttranslational modification and functional EpoR has a size between 66 and 78 kDa. A soluble form of EpoR and a truncated form of this receptor can be detected at lower molecular weight. The 59-kDa band detected by Elliott et al1 is most likely physiologically irrelevant and raises the question why proteins derived from their expression plasmid are not processed into active receptors. Third, after ligand binding, EpoR is internalized and degraded. The cancer lines used by Elliott et al1 show varying levels of EpoR surface levels under standard culture conditions. EpoR is usually detectable only after serum starvation, and lack of EpoR expression in these cells under the conditions chosen is no surprise to anyone in this research field. In addition, the C20 antibody was used at 1.32 µg/mL, whereas the manufacturer recommends 0.1 to 1.0 µg/mL. Culture conditions, which
lead to receptor internalization and degradation combined with antibody concentrations favoring unspecific binding, are inappropriate to investigate the specificity of any antibody. Fourth, mass spectrometry of trypsin-digested bands cut out of Western blots usually gives rise to many possible protein identities (IDs). HSPs bind to cytokine receptors and identification of a chaperon like HSP70 may indicate incomplete denaturing conditions. A state-of-the-art approach would have employed 2D electrophoresis instead. In the study of Elliott et al, it would have been mandatory to report probability scores for the proteins identified and we would have appreciated if the authors would have provided this data as supplementary material. Without this information, the validity of the LC-MS analysis is questionable and the results do not allow any conclusion to be drawn. Finally, the M-20 antibody is not recommended for use in immune histochemistry (IHC) by the manufacturer. Thus, immunohistochemical data provided by Elliott et al confirm the product data sheet of the M20 antibody but do not add to the knowledge on EpoR.

With the exception of solid cancer cells, the cytoprotective effect of recombinant human Epo (rhEpo) in many tissues is a widely accepted concept in the scientific community and in the pharmaceutical industry. Furthermore, an increasing number of studies show not only expression of EpoR mRNA and protein in solid cancers but also rhEpo-dependent signaling in cancer cells. Two recent clinical trials support tumor protection by rhEpo. By questioning the validity of past studies of EpoR expression in solid cancers but also rhEpo-dependent signaling in cancer cells.

By questioning the validity of past studies of EpoR expression in solid tumors, Elliott et al attempt to support the general safety of rhEpo in the treatment of cancer anemia. Unfortunately, their conclusions are not supported by the data presented and indicate methodological issues rather than novel findings.

Michael Henke, Ajay Verma, and Geza Acs

Correspondence: Michael Henke, Strahlenheilkunde Universitaetsklinikum, Robert Koch Strasse 3, Freiburg, Germany D-79106; e-mail: henke@uni-freiburg.de.

Response:

Anti-EpoR antibodies detect a 59-kDa EpoR protein

Henke and colleagues claim there are methodological problems with our study demonstrating that the 66-kDa protein detected by the C-20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) is not EpoR. We disagree with their critique of our study for 4 reasons. (1) They suggest we did not demonstrate functional expression of human EpoR encoded by our expression construct (FLAG-EpoR). However, BaF3 cells expressing FLAG-EpoR showed Epo-dependent growth, demonstrating functional expression (Janet Cheng [Amgen, Thousand Oaks, CA]; unpublished data, 2005). (2) The authors state that functional EpoR has a molecular weight of 66 to 78 kDa because of posttranslational modification. However, that statement is incorrect on theoretical grounds and is likely based on studies that used the anti-EpoR antibodies in question. The correct size is likely approximately 59 kDa. The calculated size of EpoR protein is 52.6 kDa, and the single N-linked glycosylation site would add only 3 to 4 kDa, resulting in sizes of glycosylated EpoR and FLAG-EpoR of 56 to 57 and 57 to 58 kDa, respectively (FLAG adds 1 kDa). In the Epo-responsive cell line UT-7/Epo, C-20 detected and immunoprecipitated a 59-kDa protein (Figure S2 in Elliott et al). EpoR sequences were found at 59 kDa in preparative gels, 59kDa levels decreased following EpoR shRNA treatment (Figure S1 in Elliott et al), and, although 3 different anti-EpoR antibodies detected a 59-kDa protein, they each detected larger proteins of various sizes (Figure 1). (3) Since no commercially available antibodies are suitable for detection of surface EpoR, we did not investigate surface EpoR in cancer cell lines; we reported total cellular levels. We are unaware of validated reports directly showing up-regulation of EpoR in response to serum starvation. The concentration of C-20 used in our study was 0.132 μg/mL and not 1.32 μg/mL (stated in error in our initial report). (4) We performed mass spectrometry on proteolyzed proteins isolated from preparative gels of cell extracts immunoprecipitated with C-20. The sequence analysis was rigorous. Sequence search (SEQUENT; Thermo Finnigan, San Jose, CA) was rigorous, with probability scores of cross-correlation value (XCorr) = 2.0 and delta correlation value (DelCn) = 0.12, which were used to filter false positives, and manual validation was performed on questionable spectra. More than 35% of all unique peptides (55 of 153) identified in the 66-kDa band were derived from various regions of heat-shock proteins (HSPs), and none were derived from EpoR. Specific inhibition of C-20 binding to the 66-kDa protein by an HSP70 peptide was demonstrated (Figure 1 in Elliott et al).

References

Mantle cell lymphoma (MCL) is a lymphoproliferative disorder with distinct pathologic and clinical features. Genetically, the vast majority of MCLs carry the t(11;14)(q13;q32). This characteristic chromosomal abnormality leads to deregulation of the CCND1 gene encoding cyclin D1 at 11q13 through juxtaposition with the immunoglobulin heavy chain (IGH) locus at 14q32. By gene expression profiling, 6 cases of MCL that lacked cyclin D1 mRNA expression but had a gene expression signature typical of MCL have recently been described. These cyclin D1–negative MCLs exhibited the characteristic morphologic, immunohistochemical, and clinical features of cyclin D1–positive MCL. In line with the lack of cyclin D1 mRNA expression, cyclin D1 protein was absent in these cases. Moreover, the cases did not have the t(11;14) by fluorescence in situ hybridization (FISH) analysis. Interestingly, all 6 cyclin D1–negative MCLs expressed either cyclin D2 or D3, suggesting that these cyclins can substitute functionally for cyclin D1 in MCL. Nevertheless, chromosomal aberrations affecting the CCND2 or CCND3 gene loci were not detectable in these cases by interphase FISH.

Herein, we present 2 cases of cyclin D1–negative MCL in which cyclin D2 protein expression was associated with a chromosomal translocation juxtaposing the CCND2 gene at chromosomal band 12p13 next to the IGH locus at 2p12. The lymphomas occurred in a 33-year-old woman and a 70-year-old man. Both were diagnosed with stage IV disease with bone marrow involvement. Diagnostic lymph node biopsies showed the typical morphology of MCL with a nodular and diffuse growth pattern (Figure 1A,D). Both lymphomas displayed the immunophenotype typical for MCL (CD5+, CD20+, CD10−, and CD23−), except for lack of cyclin D1 expression. Absence of the t(11;14) or variant CCND1 breaks was proven in both cases by FISH using commercially available probes (LSI IGH/CCND1, LSI CCND1 BAP; Vysis, Downers Grove, IL). Conventional cytogenetic R-banding analysis in the first case revealed the tumor cells to have the karyotype 48,XX,t(2;12)(p12;p13)+,+3,+21[11] (Figure 1B). Subsequent FISH using recently described probes1,2 showed that the t(2;12) caused breaks in the IGH and CCND2 loci, resulting in IGK-CCND2 fusion (Figure 1C insert). In the second case, metaphase analysis was not possible due to lack of suitable material. Nevertheless, interphase FISH showed breakpoints in the CCND2 and IGK loci, as well as IGK-CCND2 fusion (Figure 1E). Immunohistochemical analyses revealed strong nuclear expression of cyclin D2 protein in the majority of the tumor cells in both cases (Figure 1C,F).

Our findings indicate that cyclin D2–positive MCL can arise through juxtaposition of the CCND2 and IGK loci. Remarkably, as an asterisk, to prevent anemia in cancer patients. However, these studies have been criticized for poor study design and execution. A number of ongoing trials will determine the impact on survival of patients undergoing therapy with ESAs.

Steve Elliott, Leigh Busse, Chris Spahr, and Angus M. Sinclair

Correspondence: Steve Elliott, Amgen Inc, One Amgen Center Drive, Thousand Oaks, CA 91320; e-mail: seliott@amgen.com.

The authors are employed by Amgen Inc, a manufacturer and distributor of erythropoietic-stimulating proteins.

References

Erythropoietin receptors on cancer cells: exciting perspectives, difficult to appreciate

Michael Henke, Ajay Verma and Geza Acs