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Author Response: Effects of Fibroblastic and Endothelial Extracellular Matrices on Corneal Endothelial Cells

We thank Dr. Baum for his kind, encouraging words and for pointing out his very interesting article on how matrix influences HCEC morphology.¹ His work is of great importance to the field of corneal endothelial research. It had a major impact on our earlier work on establishing and optimizing cell cultivation and transplantation of HCECs—namely, his article, “Mass Culture of Human Corneal Endothelial Cells,”^{2,3} which we cited in one of our most important papers.³

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Bevacizumab Suppression of Establishment of Micrometastases in Experimental Ocular Melanoma

We read with great interest the article entitled, “Bevacizumab Suppression of Establishment of Micrometastases in Experimental Ocular Melanoma” by Yang et al.,¹ published in the June issue. We congratulate the authors for this informative study. However, we have a few concerns about the study.

1. In Figure 1, the authors demonstrate that bevacizumab reduced VEGF secretion. However, it is more likely that bev-

acizumab neutralized the secreted VEGF, rather than suppressed it.

2. It is not clear why, even though bevacizumab may have neutralized the secreted VEGF, the neutralized VEGF could not be measured by ELISA. The epitopes for antibodies used in ELISAs (27-191 amino acids) are likely to be against a different region of VEGF than are the functional epitopes bound by bevacizumab.

3. The authors state that VEGF secretion was slightly suppressed with 10 μ g/mL bevacizumab ($P = 0.0046$) and greatly reduced with 100 μ g/mL ($P = 0.0091$) in B16LS9 cells, compared with the effect of the IgG1 control treatment. However, they also state that the levels of VEGF after 10 and 100 μ g/mL bevacizumab were 8.51 and 15.99 pg/mL, respectively. Thus, the levels were higher in cultures treated with a higher dose of bevacizumab (100 μ g/mL). Also, in Figure 1C, it does not appear that the VEGF levels were decreased significantly by bevacizumab.

4. Several earlier studies (e.g., Ferrara et al.²) reported that bevacizumab does not neutralize mouse VEGF, raising the question of how bevacizumab may have reduced the VEGF levels.

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Author Response: Bevacizumab Suppression of Establishment of Micrometastases in Experimental Ocular Melanoma

Bevacizumab is a recombinant humanized monoclonal IgG1 antibody that binds to and inhibits the biological activity of human vascular endothelial growth factor (VEGF) in vitro and in vivo assay systems. It contains human framework regions and the complementarity-determining regions of a murine antibody that binds to VEGF.

When comparing the sequence of human VEGF protein with that of mouse VEGF protein, we found that 86% of the protein sequence in mouse VEGF164 is similar to that of human VEGF165.¹ We believe that it is possible for bevacizumab to block human and mouse VEGF. A blocking antibody is defined as an antibody that does not have a reaction when combined with an antigen, but prevents other antibodies from combining with that antigen.^{2,3} We believe that bevacizumab fits this definition and explains why the neutralized VEGF is not detected by ELISA. The human or mouse VEGF immunoassay (Quantikine; R&D Systems, Minneapolis, MN) that we used in our experiment can detect human VEGF165 and VEGF121 or mouse VEGF164 and VEGF120, but does not detect VEGF189 and VEGF206. VEGF121 and VEGF165 are diffusible proteins that are secreted into the medium. VEGF189 and VEGF206 have high affinity for heparin and

are mostly bound to heparin-containing proteoglycans in the extracellular matrix. Bevacizumab binds to and neutralizes all isoforms of VEGF-A and bioactive proteolytic fragments.

Bock et al.⁴ performed three different experimental approaches using Western blot analysis, ELISA, and Biacore (surface plasmon resonance; GE Health Care, Piscataway, NJ) analysis to assess whether bevacizumab binds to murine VEGF-A. In the Western blot analyses, recombinant murine VEGF-A164, a 24-kDa protein consisting of 164 amino acid residues, and the 19.1-kDa homologous human VEGF165, which consists of 165 amino acid residues, were used. In ELISA and the Biacore analyses, the recombinant human and the homologous murine VEGF-A were used as well. Western blot analysis showed that bevacizumab bound murine and human VEGF-A. To confirm these results, Bock et al. performed two different experimental approaches for analysis of the binding of bevacizumab to murine VEGF-A. The first assay, an ELISA, analyzed the interaction of soluble bevacizumab with VEGF-A immobilized on a microtiter plate surface. In the second assay, the surface plasmon resonance technology (Biacore) was used for the analysis of interaction of soluble VEGF-A with bevacizumab immobilized on the sensor chip surface. Both assays allow a qualitative characterization of the interaction between bevacizumab and human or murine VEGF-A. In the ELISA binding assay, bevacizumab bound to both human and murine VEGF-A; however, a 1000-fold higher bevacizumab concentration was necessary to reach a similar level of binding to murine VEGF-A compared with that needed for binding to the human protein. There was no significant binding of isotype control IgG1k to murine VEGF-A at this concentration. During the Biacore surface plasmon resonance experiments, different concentrations of human and murine VEGF-A were injected, to analyze their binding to immobilized bevacizumab. A more than 10-fold higher concentration of murine VEGF-A than of human VEGF-A was necessary to reach similar resonance units. ELISA is more sensitive than Western blot analysis. It can detect a protein of at picogram concentrations; Western blot can only detect a protein at nanogram concentrations. Studies have also indicated that bevacizumab inhibits experimental choroidal and corneal neovascularization in a mouse model,^{5,6} endothelial cell proliferation in a retinopathy of prematurity mouse model,⁷ and tumor progression in a *A/J Tsc2^{+/-}* C57BL/6 background mouse model of pancreatic tumor.^{8,9}

When we initially used bevacizumab in a mouse model to treat mouse melanoma cells, we found that it slightly decreased the tumor size and number of metastases. We then performed bevacizumab experiments with human uveal melanoma cell lines. Our experiments showed that bevacizumab had much more effect on human melanoma than on mouse melanoma. We acknowledge point three of Sharma et al. and reply that we meant that 10 and 100 $\mu\text{g/mL}$ of bevacizumab reduced VEGF levels by 8.51 and 15.99 pg/mL, respectively. Bevacizumab acts as a tumor suppressor by inhibiting angiogenesis and lymphangiogenesis. Lymphangiogenesis not only plays an important role in mediating immune reactions, but also facilitates tumor metastasis¹⁰. In our study, we investigated only the antiangiogenesis function of bevacizumab.

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Passive Letter Subset Memory and Reliability of Visual Acuity Assessment

The potential for systematic bias associated with using the best of multiple measures of visual acuity as an outcome variable has been well demonstrated.¹ The point is well made that the reliability and/or validity of assessments of changes in visual acuity might be adversely affected.¹ I would like to comment on passive letter chart subset memory as a source of bias and/or reduced reliability when visual acuity is assessed. For example, during a routine clinical examination or assessment of a research subject there are usually multiple references to letter charts that are constructed using different sequences of the same letter subset. Sometimes the same letter chart and letter configuration are used multiple times throughout an examination or period of assessment. Low levels of passive letter subset memory have been found to persist for up to 10 days after a routine clinical examination of both eyes,² and such memory may enhance performance during assessments within that period. For example, enhancement of visual acuity was demonstrated when retesting occurred within 24 hours of a single acuity measurement,^{3,4} and may have been at least partly due to passive memory of the letter subset. However, enhancement associated with passive memory could be greater when preceded by multiple measures and/or when multiple assessments occur within a shorter time frame than 24 hours. For example, when a second eye assessment immediately follows the first assessment with the same chart or the same letter subset, the finding for the second eye could be enhanced.