giogenesis (Fig. 3) *in vitro*. From these *in vitro* experiment, it is thought that HUVEC proliferation and migration were mediated exclusively via the kinase insert domain-containing receptor (KDR, also known as VEGF receptor-2) signaling pathway. On the other hand, fms-like tyrosine kinase 1 (flt-1, also known as VEGF receptor-1) activation mediated a distinctive inhibitory signaling pathway through PI-3K that downregulated cell proliferation pathway triggered by KDR (36). The induction of the expression of VEGF was stimulated by hypoxia (37), low glucose concentration (28, 38), cytokines (39), and estrogen (40). In particular, under a hypoxic condition, it has been shown that HIF-1 regulated the expression of VEGF and various hypoxia-induced proteins (41, 42). VEGF has mainly three isoforms (VEGF121, VEGF165, and VEGF189) as demonstrated by alternative splicing, and Herve *et al.* (43) have shown that VEGF165 and VEGF189 induced KDR but not flt-1 mRNA expression in HUVECs. Suzuki *et al.* (44) have shown that hypoxia-exposed HepG2 cells upregulated VEGF mRNA expression and VEGF release and hypoxia-exposed conditioned medium used for cultivating HepG2 cells induced flt-1 and KDR expressions in HUVECs. In the HUVEC proliferation and angiogenesis assays, we did not observe any significant differences between the cell cultures treated with the conditioned medium from the growth phase and from the stable phase when the assays were performed under almost the same VEGF concentration (Fig. 3). Therefore, these results suggest that the VEGF released from the RFB during the growth phase is a similar VEGF isoform to that released during the stable phase.

The expression of nine genes listed in Table 1 was induced by hypoxia in the static culture of HepG2 cells (45). However, during the RFB cultivation of HepG2 cells, the expression level of HIF-1 was not changed (data not shown). In addition, VEGF productivity under a high DO concentration did not change significantly (Fig. 2). Mizukami *et al.* (32) have shown that the hypoxic induction of VEGF expression was partially blocked in HIF-1 knock-down cancer cells; however, HIF-1 knock-down cancer cells which were tranfected to mouse maintained VEGF activity as shown by microvessel formation in xenografted cells. In addition, interleukin-8 (IL-8) expression was induced by hypoxia in HIF-1 knock-down cells via NF-kappaB activation. Bobrovniknva *et al.* (46) have shown that VEGF and IL-8 secretions were dramatically induced by glutamine deprivation in human breast carcinoma cells via NF-kappaB and activator protein-1 (AP-1) activations. Therefore, it is likely that the induction of VEGF expression in the RFB culture of HepG2 cells is induced by NF-kappaB activation. NF-kappaB could be activated by the exposure of cells to LPS or inflammatory cytokines such as TNF or interleukin-1, viral infection or viral gene products, UV irradiation, B or T cell activation, and by other physiological and nonphysiological stimuli (47). Further study is needed to clarify the mechanism of the induction of VEGF expression in the RFB cell culture system. In conclusion, the RFB cell culture system can be used to culture cancer cells with a dormant phenotype and is a useful device for clarifying the unknown mechanisms of the induction of angiogenic factors and the kinetics of tumor progression as an alternative method of tumor cell xenograft to laboratory animals.

ACKNOWLEDGMENTS

We are deeply grateful to Ms. Serizawa and Mr. Wada of ABLE Corporation for assistance in the conduct of the RFB cell cultivation and the VEGF ELISA assay, and to Mr. Ino of Nagoya University for angiogenesis analysis. This study was supported by the Program for the Promotion of Fundamental Studies in Health Sciences and a grant (Research on Health Sciences Focusing on Drug Innovation) from the Japanese Health Sciences Foundation.

REFERENCES

1. **Kawada, M., Nagamori, S., Aizaki, H., Fukaya, K., Niiya, M., Matsuura, T., Sujino, H., Hasumura, S., Yoshida, H., Mizutani, S., and Ikenaga, H.:** Massive culture of human liver cancel cells in a newly developed radial flow bioreactor system: ultrafine structure of functionally enhanced hepatocarcinoma cell lines. In Vitro Cell Dev. Biol. Anim., **34**, 109–115 (1998).
2. **Iwahori, T., Matsuura, T., Machashi, H., Sugo, K., Saito, M., Hosokawa, M., Chiba, K., Masaki, T., Aizaki, H., Ohkawa, K., and Suzuki, T.:** CYP3A4 inducible model for *in vitro* analysis of human drug metabolism using a bioartificial liver. Hepatology, **37**, 665–673 (2003).
3. **Aizaki, H., Nagamori, S., Matsuda, M., Kawakami, H., Hashimoto, O., Ishiko, H., Kawada, M., Matsuura, T., Hasumura, S., Matsuura, Y., Suzuki, T., and Miyamura, T.:** Production and release of infectious hepatitis C virus from human liver cell culture in the three-dimensional radial-flow bioreactor. Virology, **314**, 16–25 (2003).
4. **Sakai, Y., Furukawa, K., and Suzuki, M.:** Immobilisation and long-term albumin secretion of hepatocyte spheroids rapidly formed by rotation tissue culture methods. Biotechnol. Tech., **6**, 527–532 (1992).
5. **Kurosawa, H., Yuminamochi, E., Yasuda, R., and Amano, Y.:** Morphology and albumin secretion of adalt rat hepatocytes cultured on a hydrophobic porous expanded polytetrafluoroethylene membrane. J. Biosci. Bioeng., **95**, 59–64 (2003).
6. **Yamada, K., Kamihira, M., and Iijima, S.:** Enhanced cell aggregation and liver functions using polymers modified with a cell-specific ligand in primary hepatocyte cultures. J. Biosci. Bioeng., **88**, 557–562 (1999).
7. **Khaoustov, V. I., Risin, D., Pellis, N. R., and Yoffe, B.:** Microarray analysis of genes differentiatially expression in HepG2 cell cultured in simulated microgravity: preliminary report. In Vitro Cell Dev. Biol. Anim., **37**, 84–88 (2001).
8. **Hongo, T., Kajikawa, M., Ishida, S., Ozawa, S., Ohno, Y., Sawada, J., Umezawa, A., Ishikawa, Y., Kobayashi, T., and Honda, H.:** Three-dimensional high-density culture of HepG2 cells in a 5-ml radial-flow bioreactor for construction of artificial liver. J. Biosci. Bioeng., **99**, 237–244 (2005).
9. **Aguiree Ghiso, J. A., Kovalski, K., and Ossowski, L.:** Tumor dormancy induced by downregulation of urokinase receptor in human carcinoma involves integrin and MAPK signaling. J. Cell Biol., **147**, 89–104 (1999).
10. **Folkman, J.:** Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med., **1**, 27–31 (1995).
11. **Mazure, N. M., Chen, E. Y., Laderoute, K. R., and Giaccia, A. J.:** Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. Blood, **90**, 3322–3331 (1997).
12. **Shachaf, C. M., Kopelman, A. M., Arvanitis, C., Karlsson, A., Beer, S., Mandl, S., Bachmann, M. H., Borowsky, A. D., Ruebner, B., Cardiff, R. D., Yang, Q., Bishop, J. M.,**

Contag, C. H., and Felsher, D. W.: MYC inactivation uncovers pluripotent differentiation and tumor dormancy in hepatocellular cancer. Nature, **431**, 1112–1117 (2004).
13. **Hanafusa, T., Yumoto, Y., Nouso, K., Nakatsukasa, H., Onishi, T., Fujikawa, T., Taniyama, M., Nakamura, S., Uemura, M., Takuma, Y., Yumoto, E., Higashi, T., and Tsuji, T.:** Reduced expression of insulin-like growth factor binding protein-3 and its promoter hypermethylation in human hepatocellular carcinoma. Cancer Lett., **176**, 149–158 (2002).
14. **Claffey, K. P. and Robinson, G. S.:** Regulation of VEGF/VPE expression in tumor cells: consequences for tumor growth and metastasis. Cancer Metastasis Rev., **15**, 165–176 (1996).
15. **Ferrara, N. and Alitalo, K.:** Clinical application of angiogenic growth factors and their inhibitors. Nat. Med., **5**, 1359–1364 (1999).
16. **Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N.:** Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth *in vivo*. Nature, **362**, 841–844 (1993).
17. **Millauer, B., Shawver, L. K., Plate, K. H., Risau, W., and Ullrich, A.:** Glioblastoma growth inhibited *in vivo* by a dominant negative Flk-1 mutant. Nature, **367**, 576–579 (1994).
18. **Saleh, M., Stacker, S. A., and Wilks, A. F.:** Inhibition of growth of C6 glioma cells *in vivo* by expression of antisense vascular endothelial growth factor sequence. Cancer Res., **56**, 393–401 (1996).
19. **Okino, K., Konishi, H., Doi, D., Yoneyama, K., Ota, Y., Jin, E., Kawanami, O., and Takeshita, T.:** Up-regulation of growth factor receptor-bound protein 10 in cervical sequamous cell carcinoma. Oncol. Rep., **13**, 1069–1074 (2005).
20. **Suganuma, T., Ito, K., Shibata, K., Kajiyama, H., Nagasaka, T., Mizutani, S., and Kikkawa, F.:** Functional expression of the angiotensin II type 1 receptor in human ovarian carcinoma cells and its blockade therapy resulting in suppression of tumor invasion, angiogenesis, and peritoneal dissemination. Clin. Cancer Res., **11**, 2686–2694 (2005).
21. **Saarnio, J., Parkkila, S., Parkkila, A. K., Pastorekova, S., Haukipuro, K., Pastorek, J., Juvenen, T., and Kartunen, A. J.:** Transmembrane carbonic anhydrase, MN/CA IX, is a potential biomaker for biliary tumor. J. Hepatol., **35**, 643–649 (2001).
22. **Baer, S., Casaubon, L., Schwartz, M. R., Marcogliese, A., and Younes, M.:** Glut3 expression in biopsy specimens of laryngeal carcinoma is associated with poor survival. Laryngoscope, **112**, 393–396 (2002).
23. **Oehler, M. K., Fischer, D. C., Orlowska-Volk, M., Herrle, F., Kieback, D. G., Rees, M. C., and Bicknell, R.:** Tissue and plasma expression of the angiogenic peptide adrenomedullin in breast cancer. Br. J. Cancer, **89**, 1927–1933 (2003).
24. **Chakrabarti, L., Turley, H., Campo, L., Han, C., Harris, A. L., Gatter, K. C., and Fox, S. B.:** The transcription factor DEC1 (stra13, SHARP2) is associated with the hypoxic response and high tumor grade in human breast cancers. Br. J. Cancer, **91**, 954–958 (2004).
25. **Tang, Z. Y., Sun, F. X., Tian, J., Ye, S. L., Liu, Y. K., Liu, K. D., Xue, Q., Chen, J., Xia, J. L., Qin, L. X., and other 9 authors:** Metastatic human hepatocellular carcinoma models in nude mice and cell line with metastatic potential. World J. Gastroenterol., **7**, 597–601 (2001).
26. **Huynh, H., Chow, P. K., Ooi, L. L., and Soo, K. C.:** A possible role for insulin-like growth factor-binding protein-3 autocrine/paracrine loops in controlling hepatocellular carcinoma cell proliferation. Cell Growth Differ., **13**, 115–122 (2002).
27. **Lin, X., Nelson, P., and Gelman, I. H.:** SSeCKS, a major protein kinase C substrate with tumor suppressor activity, regulates G(1)→S progression by controlling the expression and cellular compartmentalization of cyclin D. Mol. Cell Biol., **20**, 7259–7272 (2000).
28. **Sone, H., Kawakami, Y., Okuda, Y., Kondo, S., Hanatani, M., Suzuki, H., and Yamashita, K.:** Vascular endothelial growth factor is induced by long-term high glucose concentration and up-regulated by acute glucose deprivation in cultured bovine pigmented epithelial cells. Biochem. Biophys. Res. Commun., **221**, 193–198 (1996).
29. **Hermes, M., Osswald, H., Mattar, J., and Kloor, D.:** Influence of an alter methylation potential on mRNA methylation and gene expression in HepG2 cells. Exp. Cell Res., **294**, 325–334 (2004).
30. **Hasebe, Y., Egawa, K., Yamazaki, Y., Kunimoto, S., Hirai, Y., Ide, Y., and Nose, K.:** Specific inhibition of hypoxia-inducible factor (HIF)-1 alpha activation and of vascular endothelial growth factor (VEGF) production by flavonoids. Biol. Pharm. Bull., **26**, 1379–1383 (2003).
31. **Jensen, R. L.:** Growth factor-mediated angiogenesis in the malignant progression of glial tumors: a review. Surg. Neurol., **49**, 189–195 (1998).
32. **Mizukami, Y., Jo, W. S., Duerr, E. M., Gala, M., Li, J., Zhang, X., Zimmer, M. A., Iliopoulos, O., Zukerberg, L. R., Kohgo, Y., Lynch, M. P., Rueda, B. R., and Chung, D. C.:** Induction of interleukin-8 preserves the angiogenic response in HIF-1alpha-dificient colon cancer cells. Nat. Med., **11**, 992–997 (2005).
33. **Payet, L. D., Wang, X. H., Baxter, R. C., and Firth, S. M.:** Amino- and carboxyl-terminal fragments of insulin-like growth factor (IGF) binding protein-3 cooperated to bind IGFs with high affinity and inhibit IGF receptor interactions. Endcrinology, **144**, 2797–2806 (2003).
34. **Dufresne, A. M. and Smith, R. J.:** The adaptor protein GRB10 is an endogeneous negative regulator of insulin-like growth factor signaling. Endocrinology, **146**, 4399–4409 (2005).
35. **Shiura, H., Miyoshi, N., Konishi, A., Wakisaka-Saito, N., Suzuki, R., Muguruma, K., Kohda, T., Wakana, S., Yokoyama, M., Ishino, F., and Kaneko-Ishino, T.:** Meg1/Grb10 overexpression causes postnatal growth retardation and insulin resistance via negative modulation of the IGF1R and IR cascade. Biochem. Biophys. Res. Commum., **329**, 909–916 (2005).
36. **Zeng, H., Dvorak, H. F., and Mukhopodyay, D.:** Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) receptor-1 down-modulates VPF/VEGF receptor-2-mediated endothelial cell proliferation, but not migration, through phophatidylinositol 3-kinase-dependent pathway. J. Biol. Chem., **276**, 26969–26979 (2001).
37. **Wenger, R. H., Rolfs, A., Marti, H. H., Bauer, C., and Gassmann, M.:** Hypoxia, a novel inducer of acute phase gene expression in human hepatoma cell line. J. Biol. Chem., **270**, 27865–27870 (1995).
38. **Park, S. H., Kim, K. W., Lee, Y. S., Baek, J. H., Kim, M. S., Lee, Y. M., Lee, M. S., and Kim, Y. J.:** Hypoglycemia-induced VEGF expression is mediated by intercellular Ca^{2+} and protein kinase C signaling pathway in HepG2 human hepatoblastoma cells. Int. J. Mol. Med., **7**, 91–96 (2001).
39. **Ren, Y., Cao, B., Law, S., Xie, Y., Lee, P. Y., Cheung, L., Chen, Y., Huang, X., Chan, H. M., Zhao, P., Luk, J., Vande Woude, G., and Wong, J.:** Hepatocyte growth factor promotes cancer cell migration and angiogenic factors expression: a prognostic marker of human esophageal squamous cell carcinomas. Clin. Cancer Res., **11**, 6170–6197 (2005).
40. **Mueller, M. D., Vigne, J. L., Minchenko, A., Lebovic, D. I., Leitman, D. C., and Taylor, R. N.:** Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptors alpha and beta. Proc. Natl. Acad. Sci. USA, **97**, 10972–10977 (2000).
41. **Semenza, G. L.:** Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu. Rev. Cell Dev. Biol., **15**, 551–578 (1999).
42. **Semenza, G. L.:** HIF-1: mediator of physiological and patho-

physiological responses to hypoxia. J. Appl. Physiol., 88, 1474–1480 (2000).
43. **Herve, M. A., Buteau-Lozano, H., Mourah, S., Calvo, F., and Perrot-Applanat, M.:** VEGF189 stimulates endpthelial cells proliferation and migration *in vitro* and up-regulates the expression of Flk-1/KDR mRNA. Exp. Cell Res., **309**, 24–31 (2005).
44. **Suzuki, H., Seto, K., Shinoda, Y., Mori, M., Ishimura, Y., Suematsu, M., and Ishii, H.:** Paracrine upregulation of VEGF receptor mRNA in endothelial cells by hypoxia-exposed Hep G2 cells. Am. J. Physiol., **276**, G92–97 (1999).
45. **Sonna, L. A., Cullivan, M. L., Sheldon, H. K., Pratt, R. E., and Lilly, C. M.:** Effect of hypoxia on gene expression by human hepatocytes (HepG2). Physiol. Genomics, **12**, 195–207 (2003).
46. **Bobrovnikova-Marjon, E. V., Marjon, P. L., Barbash, O., Vander Jagt, D. L., and Abcouwer, S. F.:** Expression of angiogenic factors vascular endothelial growth factor and interleukin-8/CXCL8 is highly responsive to ambient glutamine availability: role of nuclear factor-kappaB and activating protein-1. Cancer Res., **64**, 4858–4869 (2004).
47. **Baldwin, A. S., Jr.:** The NF-kappaB and I kappa B proteins: new discoveries and insights. Annu. Rev. Immunol., **14**, 649–683 (1996).