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CORRESPONDENCE

Proliferating infantile hemangioma promotes α-fetoprotein production by HepG2 cells

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INTRODUCTION

Infantile hemangioma (IH) affects 4–10% of children.[1–3] It is characterized by an initial rapid proliferation followed by spontaneous slow involution, often leaving a fibrofatty residuum.[4,5]

α-Fetoprotein (AFP) is a serum growth factor produced by the liver during fetal liver development/regeneration and in hepatic cancer.[6] Patients with extra-hepatic proliferating IH demonstrate elevated serum levels of AFP, which declines to normal levels during involution and following surgical excision or propranolol treatment of IH.[7] However, AFP is not expressed by IH itself.[7]

We hypothesized that IH may produce a factor responsible for the production of AFP from a secondary organ, possibly the liver. This study investigated the expression of AFP by a hepatic cell line—HepG2 cells, following co-culturing with proliferating IH-derived primary cell lines.

MATERIALS AND METHODS

Cell co-culture

Proliferating IH-derived primary cell lines from six patients with a mean age of 6 (range, 3–12) months were obtained from the Gillies McIndoe Research Institute Tissue Bank for this study, which was approved by the Central Health and Disability Ethics Committee (Ref. 13/CEN/130). Hematoxylin and eosin staining of the IH tissue samples showed the typical plump endothelial cells lining the microvessels of IH with tiny lumens (data not shown) and the expression of GLUT-1, a marker of IH.[2] A total of 1×10^5 proliferating IH-derived cells for each biological replicate and one population of human umbilical vein endothelial cell (HUVEC) line (cat# PCS-100-010, ATCC, Manassas, VA, USA) were plated out on transwell inserts (cat#CLS3470, Corning, Corning, NY, USA) and allowed to adhere for 72 h. In parallel, 1×10^5 HepG2 cells (cat#HB-8065, ATCC) were seeded onto each well of a 24-well plate (cat#TCO010024, Jet BioFil, Guangzhou, China) and allowed to adhere for 72 h. The inserts were then transferred onto a corresponding well of the 24-well plate for co-culturing. HepG2 cells were harvested at 1–4 days following co-culture. All cells were cultured in Dulbecco's modified Eagle's medium with high glucose and containing pyruvate (cat#10569010, ThermoFisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (cat#10091148, ThermoFisher Scientific), 1% penicillin–streptomycin (cat#15140122, ThermoFisher Scientific), and 0.2% gentamicin/amphotericin B (cat#R01510 ThermoFisher Scientific). HepG2 cells co-cultured with HUVECs were used as a negative control. All experiments were performed in triplicates.

Western blotting

Total protein was extracted from the HepG2 cells using RIPA Buffer (cat#R0278, Sigma-Aldrich, St Louis, MA, USA) and precipitated using the ProteoExtract Protein Precipitation Kit (cat#539180, Merck Millipore, Burlington, MA, USA). Protein was quantitated using Qubit (ThermoFisher Scientific) and total lysates were separated by 4–12% 1DE sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane using the iBlot2 (ThermoFisher Scientific). The iBind Flex (ThermoFisher Scientific) was used to probe the membrane with the primary antibodies for AFP (1:1000, cat#ab133617, Abcam, Cambridge, UK) and the housekeeping protein α-tubulin (1:1000, cat#62204, Invitrogen, Carlsbad, CA, USA), with detection using the secondary antibodies goat anti-rabbit Alexa Fluor 647 (1:2000, cat#A21244, Life Technologies) and goat anti-mouse Alexa Fluor 488 (1:2000, cat#A21202, Life Technologies). The ChemiDoc MP Imaging System (Biorad) and ImageLab 5.0 software (Biorad) were used for band detection and analysis by densitometry. The expression of AFP was normalized against the housekeeping protein α-tubulin and expressed as relative fluorescence units. The results are expressed as the average of all biological replicates, with error bars representing the standard error of the mean.

Reverse transcriptase–quantitative real-time PCR (RT-qPCR)

HepG2 cells were lysed in RLT lysis buffer (cat#79216, Qiagen, Hilden, Germany) with RNA extraction performed using the RNeasy Micro Kit (Qiagen) and QIAcube (Qiagen) and quantitated with NanoDrop 2000 (ThermoFisher Scientific). RT-qPCR was performed using the Rotor-Gene Q (Qiagen) and Rotor-Gene Multiplex RT-PCR Kit (Qiagen) for the Taqman primer probes AFP (Hs00173490_M1) and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs99999905_M1) (ThermoFisher Scientific). ΔCT values were calculated by subtracting the CT of GAPDH from the CT for AFP. The results are expressed as the average of all biological replicates, with error bars representing the standard error of the mean.

Enzyme-linked immunosorbent assay (ELISA)

The Quantikine Human AFP ELISA (cat#DAFP00, R&D Systems, Minneapolis, MN, USA) was used to quantify the levels of AFP secreted into the media at each time point, at a 1:2000 dilution in the dilution buffer supplied with the kit. Absorbance was measured using the Varioskan Flash plate reader (cat#-MIB5250030, ThermoFisher Scientific) at a wavelength of 540 nm. The results are expressed as the average of all biological replicates, with error bars representing the standard error of the mean.

Aspects of this work were presented at the 22nd International Society for the Study of Vascular Anomalies Workshop, Amsterdam, The Netherlands, May 29–June 1, 2018.

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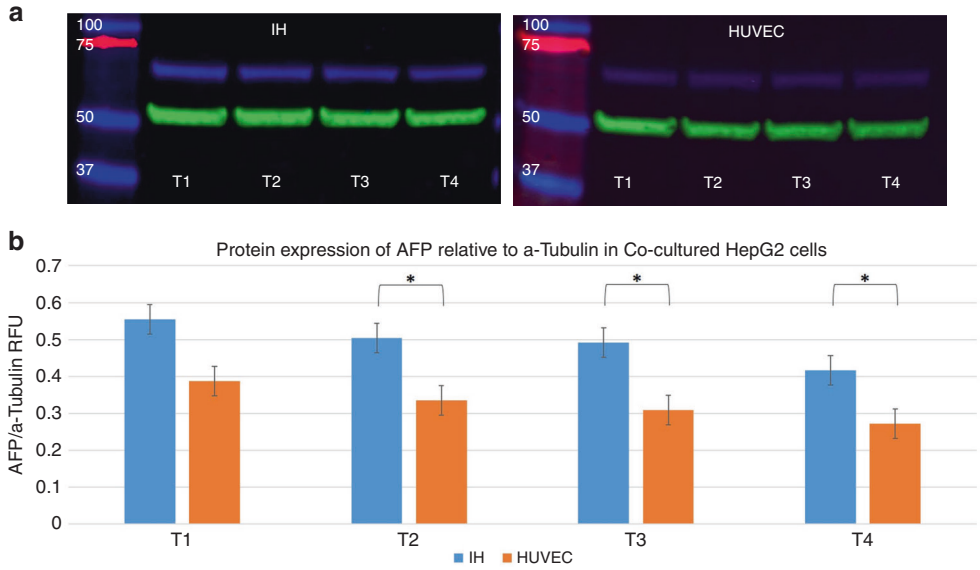

Fig. 1 Representative western blot of HepG2 cells co-cultured with six proliferating infantile hemangioma-derived primary cell lines (IH) and with human umbilical vein endothelial cells (HUVECs) at 1–4 days post co-culture, showing α-fetoprotein (AFP; **a**, blue) and the housekeeping protein α-tubulin (**a**, green). The molecular weight ladder is labeled in kDa. Densitometry analysis (**b**) of AFP/α-tubulin relative fluorescence units (RFU) showed statistically significant differences at days 2–4, relative to the HepG2 cells co-cultured with HUVECs (*$p < 0.05$)

Statistical analysis

Data obtained from the RT-qPCR, densitometry, and ELISA analyses were subjected to paired *t*-test using the SPSS version 22 software.

RESULTS

Western blotting (WB)

WB analysis of AFP production in HepG2 cells co-cultured with all six proliferating IH-derived primary cell lines demonstrated the protein expression of AFP (Fig. 1a, blue) at the expected molecular weight of 60 kDa, with approximately equal protein loading demonstrated by the housekeeping protein α-tubulin (Fig. 1a, green). Densitometry analysis (Fig. 1b) of the bands demonstrated a statistically significant increase of AFP expression at days 2 ($p < 0.05$), 3 ($p < 0.05$), and 4 ($p < 0.05$) following co-culture.

Reverse transcription–quantitative real-time PCR

Transcript expression of AFP in HepG2 cells co-cultured with all six proliferating IH-derived primary cell lines demonstrated significant average increases at days 2 ($p < 0.001$) and 3 ($p < 0.05$), relative to the HUVEC control (Fig. 2a).

Enzyme-linked immunosorbent assay

ELISA analysis showed no significant changes in the secretion of AFP between HepG2 cells co-cultured with proliferating IH-derived primary cell lines and with HUVECs, across all four time points (Fig. 2b).

DISCUSSION

The observed elevated serum levels of AFP in patients with proliferating IH and the absence of AFP in IH tissues is intriguing.[7] AFP levels are physiologically high at birth but taper to adult levels within 8–9 months;[8] however, in patients with IH these levels remain high throughout childhood and decrease upon treatment of the tumor.[7] Here we demonstrate that HepG2 cells increase the expression of AFP at both the transcriptional and translational levels in the presence of primary proliferating IH-derived cells. This novel finding would support the notion that, although IH cells do not produce/secrete AFP,[7] they may possess the ability to stimulate a secondary organ, such as the liver, to produce AFP in the affected patients.[7] The means by which this effect is achieved may be through the production of a messenger, which is released from the IH tumor and stimulates AFP production in the liver, similar to how the IH tumor secretes high levels of vascular endothelial growth factor to promote angiogenesis.[9] Identification of this putative messenger, however, is beyond the scope of this study.

We were unable to detect a statistically significant difference in the levels of secreted AFP by ELISA. This may be attributed to the relatively short duration of the experiments, therefore future research into the secretion of AFP by HepG2 cells should involve a longer time course. Furthermore, Gerets et al.[10] demonstrate metabolic discrepancies in HepG2 cells compared to primary human hepatocytes, and Wilkening et al.[11] demonstrate down-regulation of several genes in HepG2 cells compared to primary hepatocytes, particularly for the key drug-metabolizing gene family *P450*. This suggests that the HepG2 cell line may not accurately reflect hepatocyte behavior regarding AFP secretion when stimulated by IH co-culturing, therefore further research into AFP production by hepatocytes in the presence of IH cells would benefit from in vivo experiments. Elevated AFP has also been detected in yolk sac tumors and a number of non-hepatic neoplasms, as well as the normal fetal gastrointestinal tract and kidneys.[12] Therefore it is possible that IH also stimulates expression of AFP through other organs as well as in the liver, which may partly account for the lack of secretion seen in our results.

The discrepancy between expression at the transcriptional and translational levels at day 4 may be explained, in part, by the transitory delay between AFP mRNA transcriptional activation and protein translation within the cells and its subsequent secretion into the media.

This study offers novel insights into the potential use of AFP as a serological marker for measuring the response of IH to treatment, although larger studies and in vivo work is required.

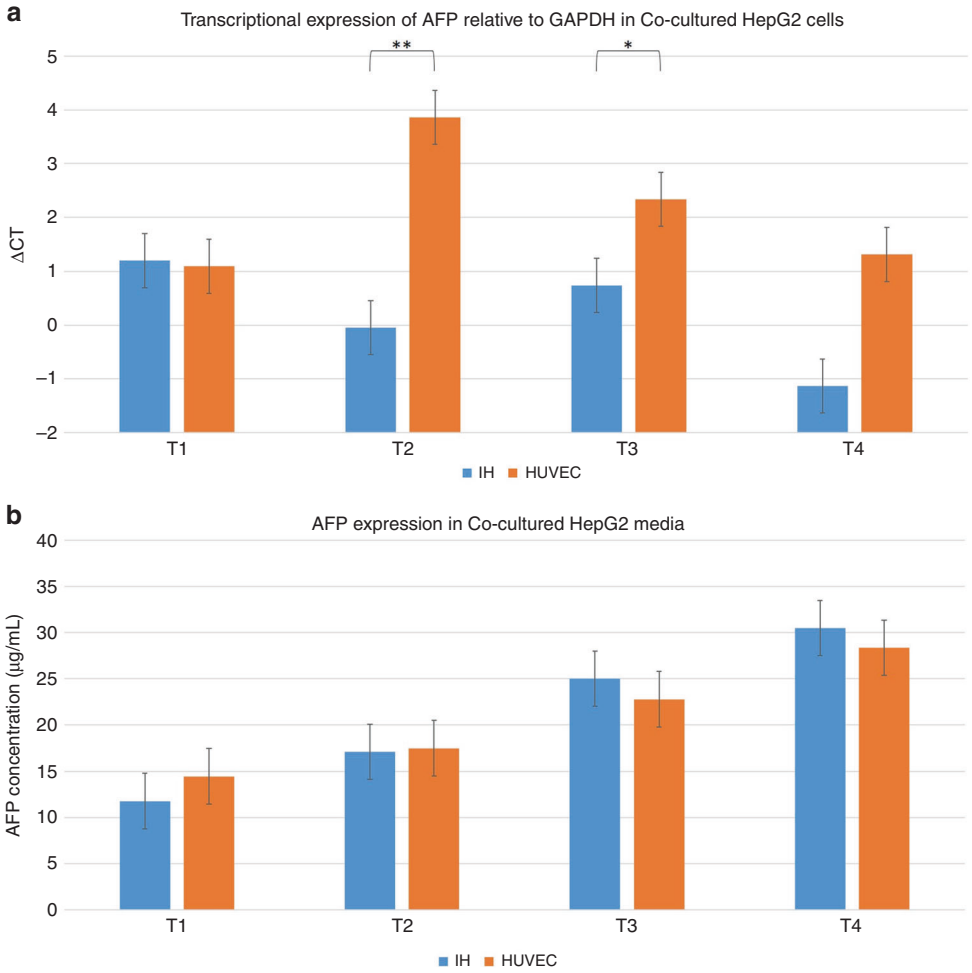

Fig. 2 **a** α-Fetoprotein (AFP) mRNA expression analysis of HepG2 cells co-cultured with six proliferating infantile hemangioma-derived primary cell lines (IH) and with human umbilical vein endothelial cells (HUVECs), at 1, 2, 3, and 4 days following co-culturing. A significant increase in AFP mRNA expression was demonstrated at days 2 and 3 in the co-cultured HepG2 cells, compared to the HepG2 cells co-cultured with HUVECs. AFP cycle threshold (CT) was compared to that of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase to produce a ΔCT value, therefore a lower ΔCT means higher mRNA expression (*$p < 0.05$; **$p < 0.001$). **b** Enzyme-linked immunosorbent assay detection of AFP in the media from HepG2 cells co-cultured with six proliferating infantile hemangioma-derived primary cell lines at days 1–4 following co-culturing, compared with the HepG2 cells grown with HUVECs

AUTHOR CONTRIBUTIONS

T.I. formulated the study hypothesis. T.I. and S.T.T. designed the study. M.M. carried out cell culture work. J.d.J. carried out the RT-PCR analysis and interpreted the results. B.v.S. carried out the WB and ELISA analyses and interpreted the results. R.M. performed the statistical analysis. B.v.S., T.I. and S.T.T. drafted the manuscript. All authors commented on and approved the manuscript.

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ADDITIONAL INFORMATION

Competing interests: T.I. and S.T.T. are inventors of a provisional patent Treatment of Vascular Anomalies (PCT/NZ2017/050032). The authors otherwise declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical statement: This study was carried out with the approval of the Central Health and Disability Ethics Committee (Ref. 13/NTB/155) with written informed consent from all subjects in accordance with the Declaration of Helsinki.

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