TECHNICAL NOTE

Myo Thaik-Oo,[1] *M.D., Ph.D.; Einosuke Tanaka,*[1] *Ph.D.; Terumasa Tsuchiya,*[2] *M.D., Ph.D.; Yoshihiko Kominato,*[3] *M.D., Ph.D.; Katsuya Honda,*[4] *M.D., Ph.D.; Kentaro Yamazaki,*[5] *M.D., Ph.D.; and Shogo Misawa,*[1] *M.D., Ph.D.*

Estimation of Postmortem Interval from Hypoxic Inducible Levels of Vascular Endothelial Growth Factor

REFERENCE: Thaik-Oo M, Tanaka E, Tsuchiya T, Kominato Y, Honda, K, Yamazaki K, Misawa S. Estimation of postmortem interval from hypoxic inducible levels of vascular endothelial growth factor. J Forensic Sci 2002;47(1):186–189.

ABSTRACT: Estimation of the postmortem interval (PMI) is one of the most important tasks in forensic medicine. Five autopsy organ tissues such as brain, lungs, heart, liver, and kidneys were taken at the time of forensic autopsy from 19 known PMI cases with a range of postmortem intervals ranging from 1 to 120 h (the mean was 25.81 h), and the time-course of vascular endothelial growth factor (VEGF) expression was measured. The human hepatoma-derived Hep 3B cell line was used as a control.

The levels of VEGF increased linearly with the PMI up to 20 h in lung ($r = 0.95$ and in kidney ($r = 0.89$), and up to 15 h PMI in liver ($r = 0.88$). The VEGF levels fell after 24 h PMI, and then remained stable. In brain, the levels of VEGF started to increase after 24 h PMI and increased linearly with PMI up to 40 h in brain ($r = 0.94$) and then begin to fall. In heart, there was no clear correlation between the PMI and VEGF level. Some variations occurred in selected cases, such as the infant and asphyxial deaths.

In conclusion, measurement of hypoxia-inducible levels of VEGF in various body organs appears to be a useful method of estimating the PMI up to 24 h in forensic medicine and pathophysiology. This method is also probably applicable in ischaemia in clinical and basic medicine.

KEYWORDS: forensic science, postmortem interval, vascular endothelial growth factor (VEGF), hypoxia, forensic science

Estimation of the postmortem interval (PMI) is one of the main issues in forensic autopsy cases. Although many forensic pathologists have tried to find a parameter for estimating the PMI, no suitable system has yet been identified. Determination of early PMI is mainly carried out using estimates of body cooling, muscular reactivity, and postmortem hypostatic staining in routine forensic practice (1). Occasionally, body cooling (rectal temperature), muscular and neuro-muscular reactivity, and body fluid biochemistry cannot be used to determine the PMI in some cases where little tissue is available, and how to determine the PMI from the remaining fragments is also an important issue in forensic practice.

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF) (2–4), is a heparin-binding glycoprotein with potent angiogenic, mitogenic and vascular permeability-enhancing activities specific for endothelial cells (5). The expression of VEGF is dramatically induced upon exposure to low oxygen tension (hypoxia or ischaemia) in a variety of cell types (6,7), and it has been suggested to play a key role in hypoxia-induced angiogenesis and in the maintenance of vascular homeostasis. Expression of VEGF increases up to 30-fold over 24 h, with an increasing duration of hypoxia (8) in the absence of glucose deprivation.

VEGF is a polypeptide growth factor, and a member of the platelet-derived growth factor family (9,10). It is a 34- to 43-kD dimeric protein, synthesized and secreted by a variety of tumor and normal cells. VEGF exists as four different homodimeric isoforms, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, generated by alternative splicing from a single VEGF gene containing eight exons (11–13) on chromosome 6p21.3. $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ are basic proteins with a high affinity for heparin, while $VEGF_{121}$ is a weakly acidic protein with no affinity for heparin (11). Cells in the body exposed to hypoxic conditions (global ischaemia) after irreversible circulatory arrest (death) exhibit increased expression of VEGF.

The induction of hypoxia-inducible factor VEGF reflects the period of hypoxia. After death, blood circulation stops, its oxygen-carrying capacity is lost, and all the body tissues become hypoxic or anoxic. In that situation, the VEGF level might reflect the duration of the hypoxemic and anoxemic condition, and we expect that it might be possible to estimate the PMI from the VEGF expression in body tissues in forensic cases including those where only fragmentary remains are available.

[1] Department of Legal Medicine, Institute of Community Medicine, University of Tsukuba, Ibaraki 305-8575, Japan.

[2] Department of Molecular Genetics, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-8510, Japan. Present address: Principal investigator, Research Project for Oxygen Response, Japan Space Forum, National Space Development Agency of Japan, Tokyo 197-0023, Japan.

[3] Department of Legal Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Toyama 930-0194, Japan.

[4] Course of Social Medicine, Osaka University Graduate School of Medicine F3, Osaka 565-0871, Japan.

[5] Tsukuba Medical Examiner's Office, Tsukuba Medical Center, Tsukuba 305-8558, Japan.

Received 16 Feb. 2001; in revised form 30 April 2001 and 14 May 2001; accepted 22 May 2001.

Copyright © 2002 by ASTM International

In the present study, we tried to estimate the PMI from VEGF expression levels of autopsy organ tissues in early postmortem cases.

Materials and Methods

1. Sample collection

   (a) ***Control samples***—The human hepatoma-derived Hep3B cell line was used as a control. Hep3B cells were cultured in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, USA).

   (b) ***Tissues samples***—Five autopsy organs tissues, brain, lungs, heart, liver, and kidneys, were taken at the time of forensic autopsy from 19 known PMI cases with a range of postmortem intervals ranging from 1 to 120 h (the mean was 25.81 h). The cases included 12 males and 7 females with ages ranging from 1 month to 74 years of age (the mean was 35 years). There was a variety of causes of death: 2 cases of stab wounds in the heart and great vessels, 2 road traffic accidents, 3 other trauma cases (contusion and laceration), 1 strangulation, 2 hangings, 1 heat stroke, 1 unknown, and 7 other disease cases (cerebral malaria, bronchopneumia, ischaemic heart disease, hypertension, vitamin deficiency, and alcoholic coma).

   Tissue samples were taken from the same location in the same organ (the right frontal lobe of the cerebrum, the middle lobe of the right lung, the apex of the heart, the lower part of the right liver, and the upper part of the right kidney) and snap-frozen in liquid nitrogen and stored at −80°C until processed further.

2. Sample preparation

   (a) ***Control samples***—Human hepatoma-derived Hep3B cells were cultured in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) under normoxic conditions in 95% air and 5% CO_2 at 37°C up to subconfluency (14,15). The cultures were allowed to continue to grow under normoxic conditions up to confluency, and the cells were then exposed to hypoxic conditions (N_2 95%, CO_2 5%) for 3, 6, 9, 18, 24, 48, and 72 h. Then, Hep3B cells were washed twice with phosphate buffered-saline (PBS), and suspended in PBS after scraping. The cells were centrifuged at 1200 × *g* for 5 min at 4°C. The cell pellets were then resuspended in lysis buffer (PBS, 1% Triton ×100, 1 m*M* phenylmethylsulfonyl fluoride: PMSF), homogenized and centrifuged at 10 000 × *g* for 20 min at 4°C. The supernatants were stored at −80°C until analysis.

   (b) ***Tissues samples***—The tissue samples stored at −80°C were thawed on ice and then homogenized in lysis buffer (PBS, 1% Triton X-100, 1 m*M* PMSF) using a homogenizer. After incubation for 30 min on ice, the homogenates were centrifuged at 10 000 × *g* for 20 min at 4°C, and supernatants were stored at −80°C until analysis. The protein levels of all samples were measured using a BCA protein assay reagent kit (Pierce, USA) and a UV spectrophotometer (UV-1600; Shimadzu, Australia) at the wavelength of 562 nm.

3. VEGF assay

   (a) ***Sample preparation***—The samples were diluted, if necessary, with deionized distilled water to obtain a protein concentration of 1 mg/mL for control cells and 10 mg/mL for tissue samples.

   (b) ***Enzyme linked immuno-sorbant assay (ELISA)***—The VEGF levels of autopsy tissues and Hep3B control samples were measured using a quantikine human VEGF immunoassay kit (R&D system, USA) and a microplate reader (InterMed, Immuno Reader NJ-2000, Japan) at wavelengths of 450 and 540 nm. A total of four measurements were made on each organ tissue. The values (coefficient of variation) obtained for each organ are as follows: lungs (4.21%, mean), kidneys (3.89%), liver (3.44%), and brain (4.33%).

4. Statistical analysis. Simple linear regression analysis was used to analyze the data and generate the equation obtained in this study.

Results

As shown in Fig. 1, we investigated the VEGF expression of the control Hep3B cells under normal oxygen conditions and prolonged hypoxic conditions up to 72 h. The level of VEGF expression increased up to 24 h under hypoxic conditions and then decreased. The increasing levels of VEGF expression in the Hep3B cells with increasing duration of hypoxia showed a linear correlation up to 24 h (Table 1). From these data, the following equation was obtained for Hep3B cells.

$$\text{Hypoxic period (h)} = 0.035 \times \text{VEGF level (ng/mL)} - 9.07$$

We then investigated the autopsy tissues from known PMI cases (Fig. 2). The results showed that the correlation between the peak levels of VEGF expression and the duration of ischaemia was different for each organ. However, the VEGF expression levels up to the peak had a linear correlation with the duration of ischaemia in all tissue samples except heart (Table 1).

As shown in Table 1, the expression levels of VEGF increased linearly with the PMI up to 20 h in the lungs and kidneys. The lev-

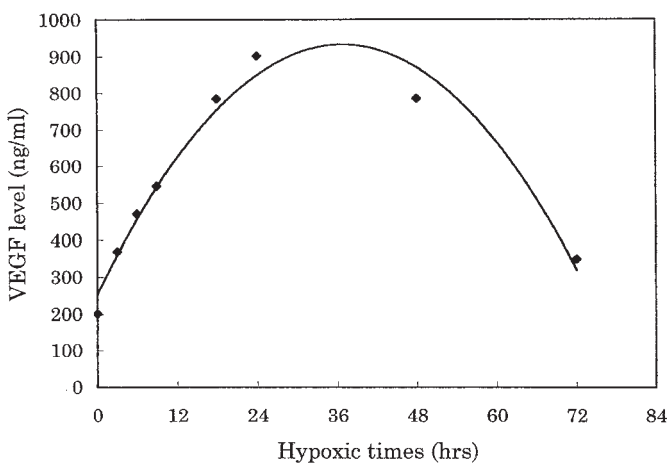

FIG. 1—*Relationship between duration of hypoxia and the VEGF levels of Hep3B cells.*

els of VEGF started to increase after a 3-h PMI and continued linearly up to a PMI of 15 h in the liver. The VEGF levels fell after a PMI of 24 h and then remained stable (Fig. 2). In the brain, the levels of VEGF started to increase after a PMI of 24 h and increased linearly up to a PMI of 42 h and then began to fall (Fig. 2). There was no clear correlation between the PMI and VEGF level in heart (data not shown).

From these results, the following equations for estimation of the PMI in autopsy tissues were obtained.

For the lungs: PMI (h) = 0.027 × VEGF levels (ng/mL) − 5.272
For the kidneys: PMI (h) = 0.019 × VEGF levels (ng/mL) − 2.082
For the liver: PMI (h) = 0.014 × VEGF levels (ng/mL) + 3.995
For the brain: PMI (h) = 0.099 × VEGF levels (ng/mL) + 24.783

Table 2 shows the VEGF levels in various organs from 19 bodies where the postmortem interval was known. There was good correlation between the PMI and VEGF level in various organs.

TABLE 1—*Differing statistical parameter of the regression line of different organs and cell line.*

Equation	*r*	Intercept (ng/mL)	Slope (ng/mL/h)
Hep3B*	0.99	266.48	27.91
Lungs†	0.95	244.85	33.84
Kidneys†	0.89	28.84	42.45
Liver‡	0.88	−120.46	56.51
Brain§	0.94	−210.91	8.80

* Up to 24 h. † Up to 20 h. ‡ Up to 15 h, § 20 to 40 h.
r = correlation coefficient.

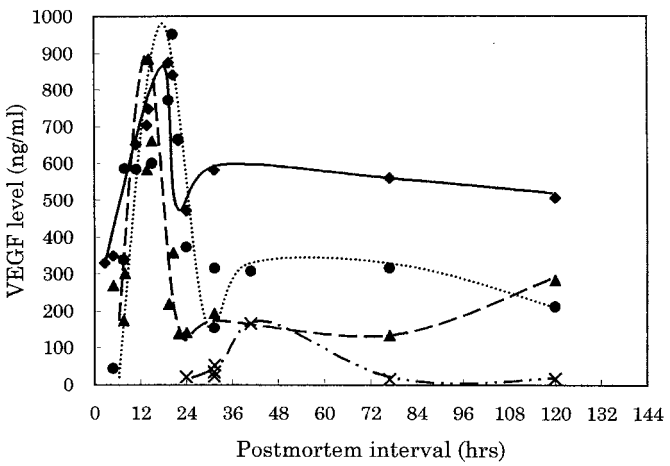

FIG. 2—*Relationship between the postmortem interval and VEGF levels of autopsy organs tissues. lungs ◆, kidneys •, liver ▲, brain X.*

Discussion

The human hepatoma-derived Hep3B cell line is used as a control because Hep3B cells have a good response to hypoxia and express VEGF and erythropoietin. The VEGF expression of Hep3B cells increased up to 24 h of hypoxia and then decreased (Fig. 1). This may be due to the fact that Hep3B cells become irresponsive to hypoxia after a 24 h period of hypoxia.

This study showed that the expression of VEGF increased linearly with the PMI up to 20 h in the lungs and kidneys, up to 15 h in the liver, and up to 42 h in the brain after irreversible circulatory arrest. After a PMI of 24 h, the levels of VEGF were downregulated and there was no significant difference among organ tissues. Therefore, PMI was estimated from the equations of the respective autopsy tissues up to 20 h in the lungs and kidneys, up to 15 h in the liver, and up to 42 h in the brain in early PMI forensic cases including those where only fragmentary remains were available. There is a difference in the linear correlation between the peak levels of VEGF expression and the duration of ischaemia in all organs except the heart. Moreover, VEGF expression patterns and peak times were the same in the lungs and kidneys (Fig. 2), and this may be due to their hypermicrovasularity (17). However, the differential expression patterns and peak times in the brain and liver may

TABLE 2—*The VEGF levels in various organs from 19 bodies where the postmortem interval was known.*

No.	Sex	Age, Year	Cause of Death	PMI, h	VEGF, ng/mL				
					Lung	Liver	Kidney	Brain	Heart
1	M	2 month	Vitamin deficiency	2.75	330	ND	ND	ND	ND
2	M	6	Bronchopneumia	4.91	350	269	44	ND	ND
3	F	34	Strangulation	7.65	ND	174	338	ND	ND
4	M	64	Ischaemic heart disease	7.91	ND	301	586	ND	ND
5	F	23	Hypertension	10.91	650	ND	584	ND	ND
6	M	48	Acute alcohol intoxication	13.75	704	582	ND	ND	ND
7	M	40	Hanging	14.25	749	884	ND	ND	ND
8	F	21	Hanging	15.1	875	660	600	ND	ND
9	F	74	Traffic accident	19.47	ND	220	771	ND	255
10	M	56	Subdural haematoma	20.62	841	357	952	ND	219
11	M	1 month	Bronchopneumia	22	661	138	666	35	175
12	M	6 month	Heat stroke	24.02	472	142	373	22	253
13	M	51	Traffic accident	31.29	582	193	155	ND	ND
14	F	UK	Subdural haematoma	31.3	ND	ND	ND	53	16
15	F	59	Haemarrhagic shock	31.39	ND	ND	316	166	158
16	M	54	Haemarrhagic shock	40.83	ND	ND	307	15	ND
17	M	55	Haemarrhagic shock	77	560	133	316	ND	ND
18	F	UK	Unknown (drawing ?)	120	506	283	212	17	ND
19	M	49	Cerebral malaria	22.75	528	449	1091	ND	ND

M = male, F = female, PMI = postmortem interval, ND = not determined, UK = unknown.

be due to the difference in their haemodynamic and haemokinetic functions (16,17). However, it will be necessary to increase the sample size and improve the correlation between the PMI and VEGF expression.

The levels of VEGF in the poisoning case and in an infant under 1 month remained steady, and the reason for this is unknown. The VEGF level is higher in the cases involving sudden and massive blood loss, strangulation and hanging, ischaemic heart disease, and cerebral malaria. It is speculated that, in asphyxia cases (e.g., strangulation and hanging), the oxygen supply to all organs is suddenly reduced, and all tissue cells become hypoxic or anoxic and expression of VEGF increases. In the case of sudden and massive blood loss and cerebral malaria, VEGF expression in hypoxic organ tissues increases because oxygen-carrying red blood cells are reduced (in sudden and massive blood loss) or damaged (in malaria). In the case of ischaemic heart disease, cardiac muscle function is reduced, leading to insufficient oxygen supply, and the tissues become hypoxic.

Compared with other common parameters, such as body cooling, muscular and neuromuscular reactivity, and postmortem hypostatic staining (lividity), used to estimate early PMI cases, our parameter is less responsive to interfering factors and is easy to sample at the time of autopsy.

The body cooling method is one of the commonly used estimation methods of PMI in early cases. The influencing factors involved in this method are: (a) body temperature at the time of death (fever, hypothermia, peripheral circulatory disorders, presence of hyperthyroidism or hypothyroidism, and emotional stress associated with pleasure and displeasure), (b) temperature difference between the body and the environmental (which can vary widely and rapidly in certain condition), (c) air movements (wind and draughts affect the body temperature by convection, conduction, and evaporation), (d) rain, humidity, and snow, (e) body posture (affects the heat loss by varying the effective exposed surface area), (f) body size (mass/surface area ratio), (g) naked, clothing or other coverings, (h) body fat (acts as an insulator), (i) cause of death (heat stroke, cold stiffness, septicaemia), and whether the body is wet or dry (1). There are other factors which affect the muscular reactivity and neuromuscular reflexes methods, such as the cause of death (heat and cold stiffness, electrocution) (18), effect of environmental temperature, influence of the central nervous system, and cadaveric spasm (instantaneous rigor) (17) and the postmortem lividity (20–22) method, such as positional changes after death. Furthermore, person-to-person variation is also a significant factor in these methods. This method has one disadvantage in that only PMI up to 24 h after death can be estimated, and this is stated in the paper.

A single sample is priced at approximately US$8.00. Basically, a quantikine human VEGF immuoassay kit and a microplate reader are required for the measurement of VEGF levels in organs and this takes about two days.

In conclusion, these results show that measurement of the levels of VEGF in four autopsy organs (brain, lung, liver, and kidney) is a good means of determining the PMI up to 24 h (early PMI) in forensic and pathophysiology cases. Additional studies are needed to verify the results reported here.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (B) (No. 10470118) from the Japan Society for the Promotion of Science.

References

1. Henssge C, Knight B, Krompecher T, Madea B, Nokes L, editors. The estimation of time since death in the early postmortem period. London: Edward Arnold. The Bath Press 1995.
2. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, et al. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest 1989;84:1470–8.
3. Connolly DT, Olander JV, Heuvelman DM, Nelson R, Monsell R, Siegel NR, et al. Human vascular permeability factor: isolation from U937 cells. J Biol Chem 1989;264:20017–24.
4. Connolly DT. Vascular permeability factor: a unique regulator of blood vessel function. J Cell Biochem 1991;47:219–23.
5. Tischer E, Mitchell R, Silva M, Gospdarowicz G, Fiddes JC, Abarhan JA. The human gene for vascular endothelial growth factor. J Biol Chem 1991;266:11947–54.
6. Shweiki D, Itin A, Scoffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 1992;359:843–5.
7. Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human glioma in vivo. Nature 1992;359:845–8.
8. Sinor AD, Irvin SM, Cobbs CS, Chen J, Graharn SH, Greenberg DA. Hypoxic induction of vascular endothelial growth factor protein in astroglial cultures. Brain Research 1998;812:289–91.
9. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, et al. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 1989;246:1309–12.
10. Conn G, Bayne ML, Soderman DD, Kwok PW, Sullivan KA, Palisi TM, et al. Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor. Proc Nat Acad Sci USA 1990;87:2628–32.
11. Houck KA, Ferara N, Winer J, Cachianes G, Li B, Leung DW. The vascular endothelial growth factor family: identification of fourth molecular species and characterization of alternative splitting of RNA. Mol Endocrinol 1991;5:1806–14.
12. Vincenti V, Cassano C, Rocchi M, Persico G. Assignment of vascular endothelial growth factor gene to human chromosome 6p21.3. Circulation 1996;93:1493–5.
13. Tischer E, Gospodarowicz D, Mitchell R, Silva M, Schilling J, Lau K, et al. Vascular endothelial growth factor: a new member of platelet derived growth factor gene family. Biochem Biophys Res Commun 1989;165: 1198–206.
14. Goldberg MA, Glass GA, Cunningham JM, Bunn HF. The regulated expression of the erythropoietin by two human hepatoma cell lines. Proc Natl Acad Sci USA 1987;84:7972–6.
15. Forsythe JA, Jiang BH, Iyar NV, Agani F, Leung SW, Koos RD, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cel Biol 1996;16:4604–13.
16. Bryan-Brown CW. Blood flow to organs: parameter for function and survival in critical illness. Crit Care Med 1988;16:170–8.
17. Wyler F. Effects of hypoxia on distribution of cardiac output and organ blood flow in the rabbit. Regional vascular response to hypoxia. Cardiology 1975;60(3):163–72.
18. Krompecher T, Bergerioux C. Experimental evaluation of rigor mortis. VII. Effect of ante- and postmortem electrocution on the evolution of rigor mortis. For Sci Int 1988;38:27–35.
19. Polson CJ, Gee DJ, editors. The essentials of forensic medicine. Oxford: Pergamon Press, 1973.
20. Suzutani T, Ishibashi H, Takatori T. Studies on the estimation of postmortem interval. 2. The postmortem lividity. Hokkaido Zasshi 1978;52: 257–67.
21. Fechner G, Koops E, Henssge C. Cessation of liver in defined pressure conditions. Z Rechtsmed 1984;93:238–97.
22. Venezis P. Assessing hypostasis by colorimetry. For Sci Int 1991;52: 1–3.

Additional information and reprint requests:
Dr. Einosuke Tanaka
Department of Legal Medicine
Institute of Community Medicine
University of Tsukuba
Tennodai 1-1-1, Ibaraki 305–8575, Japan
Tel: 81-298-53-3057
Fax: 81-298-53-3057
Email: einosuke@md.tsukuba.ac.jp