行政院及所屬各機關出國報告

（出國類別：研究）

甲狀腺癌之分子生物研究與臨床治療
副甲狀腺之冰凍保存與再植技術

服務機關：成大醫院　一般外科

出 國 人 職　　稱：主治醫師

姓　　名：洪崇傑

出國地區：美國

出國期間：90 年 8 月 16 日至 91 年 10 月 31 日

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分類號/目: J2／西醫　J2／西醫

關鍵詞:　甲狀腺癌之分子生物研究,副甲狀腺之冰凍保存

內容摘要: Clark 教授的研究室主要是進行有關甲狀腺癌的基礎研究，包括甲狀腺癌的基因表現、治療方式及癒後因子各方面的研究。實驗室裡的研究員來自於世界各國，包括加拿大、法國、日本、韓國、土耳其、沙烏地阿拉伯各國的臨床基礎研究人才，也有來自該院與其它美國醫學院的住院醫師來此做基礎研究。我這一年的研究主題主要是做有關血管內皮生長因子C (vascular endothelial growth factor-C, VEGF-C) 和甲狀腺癌的相關研究。淋巴轉移常見於大多數之惡性腫瘤，然而淋巴轉移之分子機轉至今仍不清楚。相對於刺激新血管增生之血管內皮生長因子 (VEGF)，血管內皮生長因子C被認爲可以刺激淋巴管增生。血管內皮生長因子C於1996年首先在攝護腺癌PC-3細胞株培養液被發現，它與血管內皮生長因子165有30% 的相似度，所以被認爲是血管內皮生長因子家族的一員。血管內皮生長因子C可表現在很多的人體組織，特別是在心臟、胎盤、肌肉、卵巢和小腸等組織。血管內皮生長因子 C 主要作用於血管內皮生長因子接受器VEGFR-3 (Flt-4)，而此接受器在成人組織主要存在於淋巴之內皮細胞上，所以血管內皮生長因子C被認爲是一種淋巴管增生刺激因子。研究顯示有淋巴轉移的惡性腫瘤有較高的血管內皮生長因子C表現，並發現血管內皮生長因子C 在頭頸部惡性腫瘤、食道癌、肺癌、子宮頸癌、胰臟癌、大腸癌、胃癌及攝護腺癌中與淋巴轉移與預後有極大的關聯；但此關聯並非存在於所有惡性腫瘤，所以我研究的第一個目標在於檢測是否甲狀腺癌有此類似的關係。我們嘗試用各種定性定量的方法來檢測血管內皮生長因子 C 在 mRNA及protein上面的表現，例如RT-PCR、real-time quantification RT-PCR、Western blot 和 ELISA 等方法，在這一方面我們獲得了相當的成果。不同之甲狀腺癌有不同之轉移途徑，比如乳突癌容易有淋巴轉移，然而濾泡癌卻容易沿著血行轉移。在我們的研究中發現乳突癌比其他之甲狀腺癌有較

高之血管內皮生長因子C表現；若與同一患者之正常甲狀腺組織比較，乳突癌之腫瘤組織亦有較高之血管內皮生長因子C表現，然在其他之甲狀腺癌卻無此現象。其次，我們則利用甲狀腺癌細胞株來做有關血管內皮生長因子 C 的抑制或促進因子的研究。甲狀腺刺激素已被證實會刺激甲狀腺癌的生長和轉移，抑制甲狀腺刺激素可以控制甲狀腺癌的再發與轉移。Soh 等人曾報告甲狀腺刺激素可以藉由刺激血管內皮生長因子之分泌促進新血管增生而刺激甲狀腺癌的生長，然而甲狀腺刺激素對於淋巴增生因子(血管內皮生長因子C)之作用仍未曾被報告過。在此方面的研究，我們學到了細胞株培養的方法、細胞株傳遞的方法、細胞株治療的方法以及如何從細胞株中萃取核醣核酸和蛋白質、如何測定核醣核酸和蛋白質的濃度及如何以上述的方法來檢測血管內皮生長因子 C 的表現。

本文電子檔已上傳至出國報告資訊網

摘要

Clark 教授的研究室主要是進行有關甲狀腺癌的基礎研究，包括甲狀腺癌的基因表現、治療方式及癒後因子各方面的研究。實驗室裡的研究員來自於世界各國，包括加拿大、法國、日本、韓國、土耳其、沙烏地阿拉伯各國的臨床基礎研究人才，也有來自該院與其它美國醫學院的住院醫師來此做基礎研究。我這一年的研究主題主要是做有關血管內皮生長因子 C (vascular endothelial growth factor-C, VEGF-C) 和甲狀腺癌的相關研究。淋巴轉移常見於大多數之惡性腫瘤，然而淋巴轉移之分子機轉至今仍不清楚。相對於刺激新血管增生之血管內皮生長因子 (VEGF)，血管內皮生長因子 C 被認為可以刺激淋巴管增生。血管內皮生長因子 C 於 1996 年首先在攝護腺癌 PC-3 細胞株培養液被發現，它與血管內皮生長因子 165 有 30% 的相似度，所以被認為是血管內皮生長因子家族的一員。血管內皮生長因子 C 可表現在很多的人體組織，特別是在心臟、胎盤、肌肉、卵巢和小腸等組織。血管內皮生長因子 C 主要作用於血管內皮生長因子接受器 VEGFR-3 (Flt-4)，而此接受器在成人組織主要存在於淋巴之內皮細胞上，所以血管內皮生長因子 C 被認為是一種淋巴管增生刺激因子。研究顯示有淋巴轉移的惡性腫瘤有較高的血管內皮生長因子 C 表現，並發現血管內皮生長因子 C 在頭頸部惡性腫瘤、食道癌、肺癌、子宮頸癌、胰臟癌、大腸癌、胃癌及攝護腺癌中與淋巴轉移與預後有極大的關聯；但此關聯並非存在於所有惡性腫瘤，所以我研究的第一個目標在於檢測是否甲狀腺癌有此類似的關係。

我們嘗試用各種定性定量的方法來檢測血管內皮生長因子 C 在 mRNA 及 protein 上面的表現，例如 RT-PCR、real-time quantification RT-PCR、Western blot 和 ELISA 等方法，在這一方面我們獲得了相當的成果。不同之甲狀腺癌有不同之轉移途徑，比如乳突癌容易有淋巴轉移，然而濾泡癌卻容易沿著血行轉移。在我們的研究中發現乳突癌比其他之甲狀腺癌有較高之血管內皮生長因子 C 表現；若與同一患者之正常甲狀腺組織比較，乳突癌之腫瘤組織亦有較高之血管

內皮生長因子 C 表現，然在其他之甲狀腺癌卻無此現象。

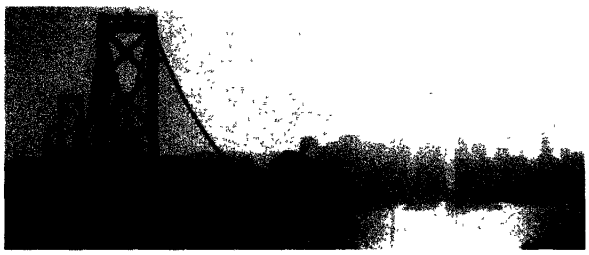
其次，我們則利用甲狀腺癌細胞株來做有關血管內皮生長因子 C 的抑制或促進因子的研究。

甲狀腺刺激素已被證實會刺激甲狀腺癌的生長和轉移，抑制甲狀腺刺激素可以控制甲狀腺癌的再發與轉移。Soh 等人曾報告甲狀腺刺激素可以藉由刺激血管內皮生長因子之分泌促進新血管增生而刺激甲狀腺癌的生長，然而甲狀腺刺激素對於淋巴增生因子(血管內皮生長因子 C)之作用仍未曾被報告過。

在此方面的研究，我們學到了細胞株培養的方法、細胞株傳遞的方法、細胞株治療的方法以及如何從細胞株中萃取核醣核酸和蛋白質、如何測定核醣核酸和蛋白質的濃度及如何以上述的方法來檢測血管內皮生長因子 C 的表現。

目次

90 年度出國報告正文

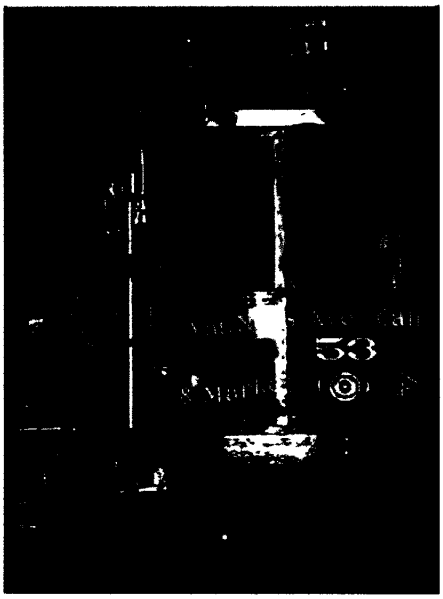


楔子

民國 90 年初得知幸運獲得教育部九十年度出國進修研究計畫，可以前往美國研究一年。隨即與日本東京女子醫科大學內分泌外科主任 Obara 教授聯絡(四年前曾獲武田獎學金前往研修四個月)，前後經過幾次的討論，最後經過他的推薦與建議，決定前往美國加州大學舊金山分校(UCSF)內分泌外科的 Clark 教授門下研究。經過幾次的書信往來，Clark 教授欣然同意我前往，Clark 教授在國際內分泌外科的領域素有盛名，不管是在基礎的研究或是臨床醫學上皆有崇高的地位與傑出的表現，並曾擔任國際內分泌外科醫學會的主席與祕書長多年，對這個領域的發展有極大的貢獻。而加州大學舊金山分校是整個加州大學體系下一個非常有名的分校，它以鼎盛的研究風氣聞名全球，並屢次在全美各醫學院的研究與教學服務的評比上名列前茅。而舊金山位居北美洲西海岸一個多丘

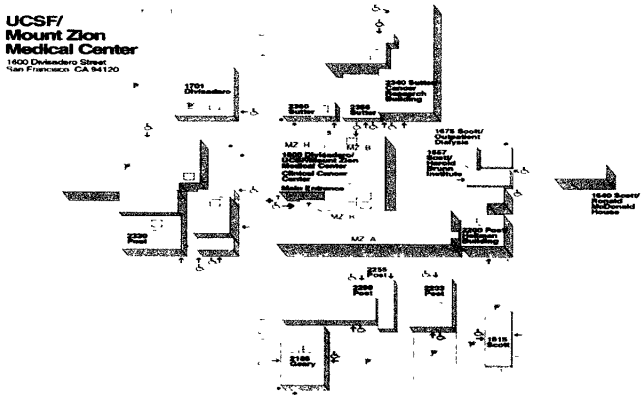
聞名遐邇的
舊金山大橋

半島的頂端，是全世界最具有特色的城市之一。舊金山是個有多元文化的城市，有渴望自由社會的各國移民、有渴望性別開放的男女同志、也有渴望科技淘金的矽谷人，形成了今天無比豐富的舊金山文化。



舊金山有名的 cable car

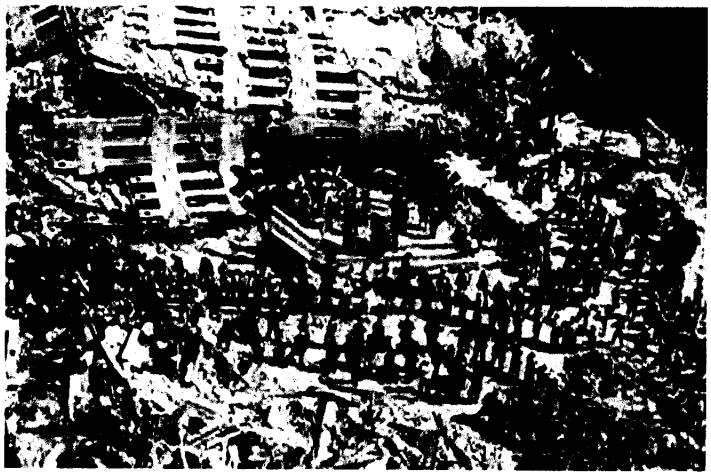
經過繁複的出國申請手續後，終於在民國 90 年八月中旬抵達了這個夢寐以求的舊金山。經過幾個星期的安頓後，接來了家人，也開始了這一年的研究之旅。加州大學舊金山分校除了在 Parnassus Height 的校本部外，在整個舊金山市內有著大大小小數十個研究中心和附屬醫院，包括 Mount Zion Medical Center、



Mount zion
Medical Center

San Francisco General Hospital、Veterans Affairs Medical Center 和 UCSF Cancer Center 等等。Clark 教授是 Mount Zion Medical Center 的外科主任，所以我這一年的研究主要也就是在 Mount Zion Medical Center 的實驗室裡度過，而加州大學舊金山分校的研究重心 UCSF Cancer Center 正位於 Mount Zion Medical Center 對面，常舉行各種研究討論會，也我也在那裡得到相當多的幫助與指導。

到舊金山不到一個月便遇到了舉世震驚的美國紐約帝國大廈的恐怖攻擊事件，這個恐怖攻擊事件與之後的炭疽病毒散播事件給整個美國社會帶來了無比的衝擊，不但讓我們見識到了美國社會如何從哀傷中復原的那股精神，也無形中讓我更專注於實驗室之研究。



受到恐怖攻擊的世界貿易大樓 Ground zero

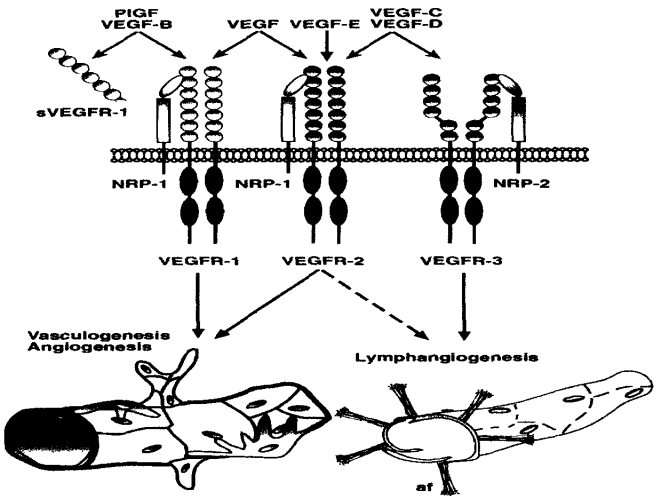
基礎研究

Clark 教授的研究室主要是進行有關甲狀腺癌的基礎研究，包括甲狀腺癌的基因表現、治療方式及癒後因子各方面的研究。實驗室裡的研究員來自於世界各國，包括加拿大、法國、日本、韓國、土耳其、沙烏地阿拉伯各國的臨床基礎研究人才，也有來自該院與其它美國醫學院的住院醫師來此做基礎研究。大家雖有著不同歷史文化背景，但在實驗室裡皆能互相支援幫忙，相處甚是融洽，這也是我此行的另一收穫。

我這一年的研究主題主要是做有關血管內皮生長因子 C (vascular

endothelial growth factor-C, VEGF-C) 和甲狀腺癌的相關研究。(關於血管內皮生長因子 C，我們已寫成 review 文章正發表中，見[redacted])

淋巴轉移常見於大多數之惡性腫瘤，然而淋巴轉移之分子機轉至今仍不清楚。相對於刺激新血管增生之血管內皮生長因子 (VEGF)，血管內皮生長因子 C 被認為可以刺激淋巴管增生。血管內皮生長因子 C 於 1996 年首先在攝護腺癌 PC-3 細胞株培養液被發現，它與血管內皮生長因子 165 有 30% 的相似度，所以被認為是血管內皮生長因子家族的一員。血管內皮生長因子 C 可表現在很多的人體組織，特別是在心臟、胎盤、肌肉、卵巢和小腸等組織。血管內皮生長因子 C 主要作用於血管內皮生長因子接受器 VEGFR-3 (Flt-4)，而此接受器在成人組織主要存在於淋巴之內皮細胞上。表現血管內皮生長因子 C 之轉基因老



鼠皮膚會有淋巴增生，合成之血管內皮生長因子 C 在雞之絨毛膜上亦會造成淋巴增生，所以血管內皮生長因子 C 被認為是一種淋巴管增生刺激因子。研究顯示有淋巴轉移的惡性腫瘤有較高的血管內皮生長因子 C 表現，並發現血管內皮生長因子 C 在頭頸部惡性腫瘤、食道癌、肺癌、子宮頸癌、胰臟癌、大腸癌、胃癌及攝護腺癌中與淋巴轉移與預後有極大的關聯；但此關聯並非存在於所有惡性腫瘤，所以我研究的第一個目標在於檢測是否甲狀腺癌有此類似的關係。

我們嘗試用各種定性定量的方法來檢測血管內皮生長因子 C 在 mRNA 及 protein 上面的表現，例如 RT-PCR (**附錄 1**)、real-time quantification RT-PCR (**附錄 2**)、Western blot (**附錄 3**) 和 ELISA 等方法，在這一方面我們獲得了相當的成果。不同之甲狀腺癌有不同之轉移途徑，比如乳突癌容易有淋巴轉移，然而濾泡癌卻容易沿著血行轉移。在我們的研究中發現乳突癌比其他之甲狀腺癌有較高之血管內皮生長因子 C 表現；若與同一患者之正常甲狀腺組織比較，乳突癌之腫瘤組織亦有較高之血管內皮生長因子 C 表現，然在其他之甲狀腺癌卻無此現象。(關於此結果，我們已寫成文章正發表中，見**附錄 9**)

其次，我們則利用甲狀腺癌細胞株來做有關血管內皮生長因子 C 的抑制或促進因子的研究。

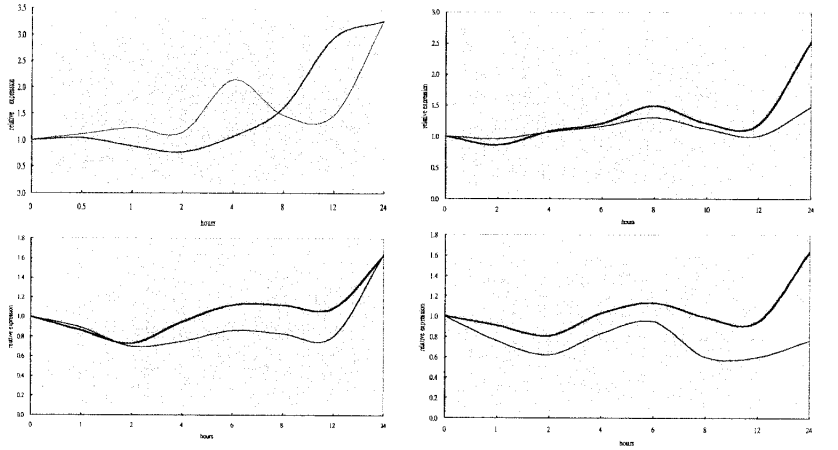
血管內皮生長因子 C 之核糖核酸表現會因各種不同生長因子之影響而上調，例如血小板血管生長因子、上皮生長因子、變形生長因子 β 和腫瘤促進素 phorbol myristate 12,13-acetate；干擾素 1β 和腫瘤壞死因子 α 在人類肺部纖維母細胞和臍靜脈內皮細胞上也會上調血管內皮生長因子 C 之核糖核酸表現。

甲狀腺刺激素已被證實會刺激甲狀腺癌的生長和轉移，抑制甲狀腺刺激素可以控制甲狀腺癌的再發與轉移。Soh 等人曾報告甲狀腺刺激素可以藉由刺激血管內皮生長因子之分泌促進新血管增生而刺激甲狀腺癌的生長，然而甲狀腺刺激素對於淋巴增生因子(血管內皮生長因子 C)之作用仍未曾被報告過。

在此方面的研究，我們學到了細胞株培養的方法、細胞株傳遞的方法、細胞株治療的方法(**附錄 4**)以及如何從細胞株中萃取核醣核酸(**附錄 5**)和蛋白質(**附錄 6**)、如何測定核醣核酸(**附錄 5**)和蛋白質的濃度(**附錄 6**)及如何以上述的方法來檢測血管內皮生長因子 C 的表現。

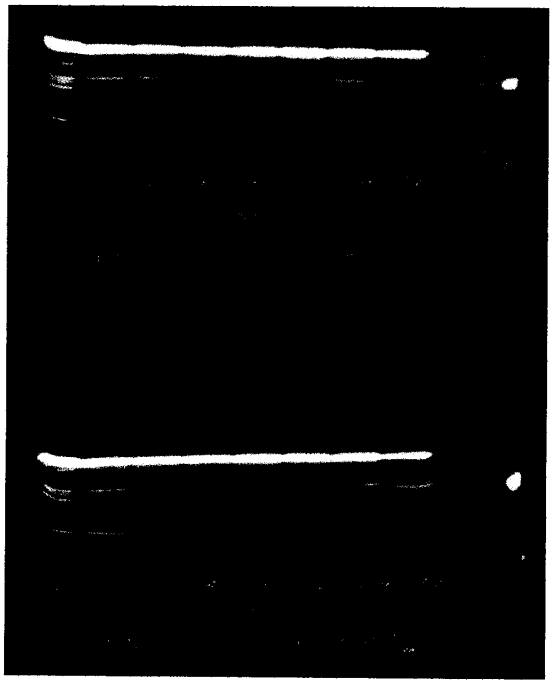
以下是我們有關"甲狀腺刺激素在甲狀腺癌細胞株上對血管內皮生長因子 C 分泌的影響"部分結果

VEGF-C expression in TPC-1
(by real time RT-PCR)



TSH ? 0 mU/ml ? 10 mU/ml

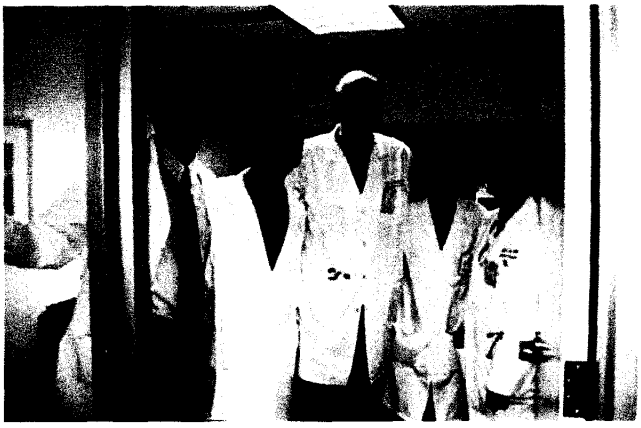
(by Western Blot)



臨床觀摩

一年的實驗室研究完畢以後，與 Clark 教授商量，決定再花三個月的時間到臨床去觀摩。Clark 教授的臨床工作主要是在 Mount Zion Medical Center 進行，大部分的病例都是屬於甲狀腺和副甲狀腺各種疾病的處理；至於腎上腺的病例則主要是由 Duh 教授在 Parnassus Height 進行。

Clark 教授除了在基礎研究與臨床服務的傑出表現，對於醫學教育也相當重視。每個禮拜四一整天的門診，一次都利用四個診間先由住院醫師或是 fellow 先負責病情的詢問及例行的檢查，並訂出初步的治療計劃；然後再由 Clark 教授一一的教學與診治。此種以教學領導門診的看病方式與國內門診形式有相當程度的差異，以臺灣現有的醫療環境短期內恐怕難以實施。另外，在每週固定



與 Clark 教授（中）和其臨床 fellow 於門診合影（左二為筆者）

的討論會上，不但注重各個特殊案例的討論，更注重整個治病思考邏輯的養成，他常常培養學生對於各種不同治療方式的正反辯證而建立起全方位的治療計劃。在臨床治療的上面更是走在世界的前端，比如在對於副甲狀腺機能亢進的病人採用 seastamibi scan 來做術前的定位，而在術中用 quick PTH assay 來判定手術的完整與否，更比如在腹腔鏡腎上腺摘除術上引用機器人(robotic operation)來進行手術，這都是我們可以努力的目標。

另外，重要的是在此期間也觀摩學習到此行的第二個目標--副甲狀腺之冰凍保存與再植技術(**附錄 7**)

後言

民國 91 年秋天，在過完 Hollowen 的 trick or treat 後，一家人帶著滿懷的感謝與美好的回憶，趕在美國誓言要剷除伊拉克海珊政權前回到魂縈夢牽的福爾摩莎。要感謝的人很多——科部主任與同事，謝謝他們在這段期間的支持與分勞；加州大學舊金山分校 Mount Zion Medical Center 的 Clark 教授、Veterans Affairs Medical Center 的 Duh 教授、UCSF Cancer Center 的 Ginzinger 教授、UCSF 移植科的 Freise 教授、UCSF 泌尿科的 Lin 教授，謝謝他們在這段期間的指導；實驗室內同甘共苦的弟兄們 JinWoo、Micheal、Nobu、Hajime、Raza、Maha、Laurent、Guiting、Wong，沒有你們的鼓勵與扶持，我無法熬過這段期間。

在 Clark 教授(右三)家舉行的 party，參加者多為 research fellow，左一為 Duh 教授。

Cancer Center 的 Ginzinger 教授(左一)和 Mamie 小姐，在 real time RT-PCR 給予我很多的指導。

RT protocol

Complementary DNA (cDNA) preparation

** In a 0.5 ml PCR tube, prepare 1.0 μg of RNA in aquanase-free water

** total volume : 7.5 μl for *5x RT buffer*, 9.5 μl for *10x RT buffer*

- dilute mRNA to 1.0 μg / μl

RNA sample	Orig conc (μg / μl) A	Orig vol (μl) B	added aquanase-free water vol (μl) (A-1)*B	Final conc (μg / μl)=1 A*B/B+(A-1)*B

- add **6.5 μl** aquanase-free water (***5x** RT buffer)*

or

add 8.5 μl aquanase-free water (*10x RT buffer)*

- add 1 μl mRNA (1.0 μg / μl)
- denature samples (including control RNAs) @ program # 10

** program # 10 -- 90 °C , 10 mins → 4 °C

** yes (retrive existing program) -- program #10 – enter -- yes -- no (printer)

- Prepare cDNA MsterMix (while samples are denaturing)

	1x	nx
25 mM $MgCl_2$	4.0	
AMV RT 5x buffer	4.0	
10 mM dNTP Mix	2.0	
RNasin	0.5	
oligo $(dT)_{12-18}$ primer	1.0	
AMV enzyme	1.0	
	12.5	

or

	1x	nx
25 mM MgCl2	4.0	
AMV RT 10x buffer	2.0	
10 mM dNTP Mix	2.0	
RNasin	0.5	
oligo $(dT)_{12-18}$ primer	1.0	
AMV enzyme	1.0	
	10.5	

** keep the final concentration of RT buffer as 1X

** prepared sample no = sample no + 1 positive control + 1 negative control + additional 1 (for pipetting error)

** RNasin : RNase inhibitor

** AMV enzyme : AMV Reverse Transcriptase

- Vortex cDNA MasterMix, Aliquot 12.5 (5x RT buffer) or 10.5 (10x RT buffer) μl of MasterMix to each.5 ml PCR tube in PCR machine

  ** pause -- enter -- open machine -- add mastermix

- RT cycle at Program #11

  ** program # 11 : (42 °C , 15 min → 90 °C , 10 min → 4 °C)

  ** store cDNA at -20 °C after RT cycle

PCR protocol (VEGF-C)

VEGF-C primer

vegf-c forward	5'-AATGTGGGGCCAACCGAGAA-3'	108.05 nmol
vegf-c reverse	3'-GCAACACAGGGAAGTATAACC-5'	113.81 nmol

1 μM = 1 μmol/L = 1000 nM = 1000 nmol/L

1st dilution (stock) (stored at -20 °C)

vegf-c forward

108.05 nmol / 108.05 μL = 108.05 x 10^{-3} μmol / 108.05 x 10^{-6} L = 1000 μM

108.05 nmol / **1080.5 μL** = 108.05 x 10^{-3} μmol / 108.05 x 10 x 10^{-6} L = **100 μM**

vegf-c reverse

113.81 nmol / 113.81 μL = 113.81 x 10^{-3} μmol / 113.81 x 10^{-6} L = 1000 μM

113.81 nmol / **1138.1 μL** = 113.81 x 10^{-3} μmol / 113.81 x 10 x 10^{-6} L = **100 μM**

2nd dilution (stored at -20 °C)

vegf-c forward and vegf-c reverse

1 μL 100μM forward primer in 19 μL aquanase-free water

5 μL 100μM forward primer in **95 μL** aquanase-free water

100μM x 1/20 = **5μM**

VEGF-C PCR Procedure

- Prepare MasterMix

	1x (VEGF-C)	6x (VEGF-C)	1x (β-actin)	6x (β-actin)
10x PCR Buffer II	5.00	30.00	5.00	30.00
25mM $MgCl_2$	5.00	30.00	5.00	30.00
10mM dNTP Mix	1.00	6.00	1.00	6.00
5' primer (μM)	4.00	24.00	1.00	6.00
3' primer (μM)	4.00	24.00	1.00	6.00
Aquanase free water	28.75	172.50	34.75	208.50
TAQ	0.25	1.50	0.25	1.50
	48.00	288.00	48.00	288.00

- liquot 48 μl of MasterMix to each 0.5 ml PCR tube
- Add 2.0 μl of cDNA
- Add 1-2 drops mineral oil
- Cycle at program #21 #46

Cycling conditions:

1st trial : 01/09/02 (program #21)

94 °C , 5 mins

94 °C , 30 secs -- 55 °C , 30 secs -- 72 °C , 1min --- 35 cycles

72 °C , 5 mins

2nd trial : 01/15/02 (program #46)

94 °C , 5 mins

94 °C , 30 secs -- 59 °C , 30 secs -- 72 °C , 1min --- 35 cycles

72 °C , 5 mins

Electrophoresis of the PCR product

- Prepare gel bed by sealing ends with scotch tape

  ** be sure to fold one end of the tape

- Preparation of 1.5 % Agarose gel

  ▲ For 10x TBE buffer

  Add 0 .75 gm Agarose, 45 ml dd-H_2O for small gel (12 wells)

  Add 1.5 gm Agarose, 90 ml dd-H_2O for large gel (20 wells)

  OR

  ▲ For 20x TBE buffer

  Add 0 .75 gm Agarose, 47.5 ml dd-H_2O for small gel (12 wells)

  Add 1.5 gm Agarose, 95 ml dd-H_2O for large gel (20 wells)

  ** now we use 10x TBE buffer in our lab

- Mix

- Microwave until the agarose is completely dissolved

** time cook → 3:00 → start

- Mix, when cool to touch,

  ▲ For 10x TBE buffer

  Add 5ml 10x TBE buffer and 5 µl of ethidium bromide (small gel)

  Add 10 ml 10x TBE buffer and 10 µl of ethidium bromide (large gel)

  OR

  ▲ For 20x TBE buffer

  Add 2.5ml 20x TBE buffer and 5 µl of ethidium bromide (small gel)

  Add 5ml 20x TBE buffer and 10 µl of ethidium bromide (large gel)

  ** the stock Ethidium bromide concentration is 10 mg/ml stock

  ** the total amount for small gel is 50 ml, and for large gel is 100 ml

- let cool, then pour, making sure the comb don't touch the gel bed
- load well using 10-15 µl of DNA sample and 3-5 µl of loading buffer (6x)
- load 5-10 µl DNA molecular marker v 8-587 bp

bp
587-540-502-458-434
267-234-213-(192-184)
(124-123)-104-89

- run 50-100 volts for 1-1.5 hour, making sure that the loading buffer runs to a distance of 3/4 of the gel

  ** be sure the electrode is from black to red

  ** electrophoresis setting : volt, time and program at S1

- Photograph

Real-time quantification RT-PCR (附錄 2)

- Prepare masterMix (126 – ((6+1)*(16+2)) for 96 wells)
- Mix by inversion, centrifuge, keep out of the light
- Prepare P/P Mix (51(46 + 5) for each VEGF-C and GUS), keep out of the light

  ** dilute the probe with aquanase free water

  ** mix the equal amount of forward and reverse primer
- Add 210 (30*7) µl MasterMix into 15 eppendorf tubes (1.5 ml)
- Add rest MasterMix into another eppendorf tube (for negative control)
- Add 70 (10*7) µl cDNA into 15 eppendorf tubes (1.5 ml)

  ** 7 = 3VEGF-C + 3GUS + 1pipette error
- Mix, centrifuge
- Add 40 µl of mixture (30 µl MasterMix + 10 µl cDNA) into 96 wells from one side of wells

  ** oblique corner should be in the right superior corner

  ** using automatic pipette
- Add 40µl MasterMix (without cDNA) into 2 control wells
- Add 10 µl P/P Mix into 96 wells from another side (to avoid contamination)
- Cover with optical adhesive cover
- Compression with compression pad
- Centrifuge at centrifuge 5403

  ** close →start→ (9) in the screen → stop
- Go to real time PCR machine

  Put another yellow cover on plate, remove base, put plate in PCR machine, tighten the switch

Western Blot for VEGF-C (H-190)

Day 1

- Thaw sample, marker, 2x sample buffer, 10% AP → keep on ice

Gel preparation

- Rinse gel plate, ethanol wash the side facing the gel and let dry
- Assemble gel plate, check leakage by dd-H_2O, marking plate 0.5 cm below the comb bottom

Separating gel preparation

** Based on protein molecular weight, determine acrylamide concentration (**8 %**) (by Gel Selection Guide) **

- In 50 ml centrifuge tube,
  add **7.25 ml** *dd-H_2O* → **3.75 ml** *4x Tris CI/SDS,* ***PH 8.8*** → **4 ml** *30% acrylamide*, mix by inversion
- Add **10 *µl*** *TEMED* and **50 *µl*** *10% AP (ammonium persulfate),* mix, quick load with pasteur pipet to the marking line
  ** total volume needed for 1 gel is about 5 ml, 2 gels is about 10 ml **
- Add small amount of n-butanol
- Wait for gel polymerization (about 70 mins)
  - ▲ prepare sample mixture (add 2x sample buffer and PBS)
  - ▲ prepare marker 5 µl (kaleidoscope prestained standards)
  - ▲ dilute 10x EB (electro buffer) to 1x EB, (100 ml 10x EB + 900 ml dd-H_2O)
  - ▲ set heater at high temperature level 8 (set at 100 °C)
- Pour n-butanol out, wash with dd-H_2O, suck H_2O with Whatman paper

Stacking gel preparation

- In 15 ml centrifuge tube, add ***3.05 ml*** *dd-H_2O* → ***1.25 ml*** *4x Tris CI/SDS,* ***PH 6.8*** → ***0.65 ml*** *30% acrylamide*, mix by inversion
- Add ***5μl*** *TEMED* and ***25μl*** *10% AP,* mix
- Quick loading on the top of the separating gel with pasteur pipet to top of plate
- Put comb in, keep at the central position, wait for polymerization (about 25 min)

  ▲ prepare sample mixture (add sample)
- Pull comb out, take assembly apart, rinse gel well with dd-H_2O

Sample preparation

- Thaw samples and marker, mix, keep on ice
- In 0.5 ml eppendorf tube, add in order : **sample buffer (2x) → PBS → sample**

  sample buffer (2x) ---- 12.5 μl

  PBS + sample ---- 12.5 μl

  ** 25 μg of protein sample added (volume calculated by protein concentration)*

Protein electrophoresis

- Assemble electrophoresis unit
- Pour **1x** Electro buffer in inner chamber, to the level above the well
- Heat sample mixture and marker at 100 °C for 3 min
- Spin down sample mixture
- Loading sample mixture (25 μl) and marker (5 μl) on gel well using gel loading pipet tip

  ** marker should be loaded on the right side

  ** empty well is better to be loaded by sample buffer
- Electrophoresis :

  Volt : 250 -- Current : 40 -- Time : 0.6 -- Prog : T/V-H --- Keep current constant

** electrophoresis time depends on the progression of the sample buffer and marker **

Protein transfer

- Prepare 3 trays : add methanol, dd-H_2O and transfer buffer
- Take PVDF membrane, soak in

  methanol : **20 sec** → dd-H_2O : **2 min** → transfer buffer
- Wet the transfer unit with transfer buffer
- Wet 3 pieces of Whatman papers, place it on the unit
- Take gel out by breaking apart two pieces of glass, using spacer as a lever
- Cut out the stacking gel and right upper angle of the separating gel by razor
- Gel will come off easily after gentle shaking in transfer buffer
- Line up the gel with PVDF membrane with the marker on the right (gel upper and membrane lower)
- Put this on the top of the Whatman paper
- Put 3 more Whatman papers soaked with transfer buffer above the gel

  ** order from top : 3 Whatman papers

  ↓

  GEL

  ↓

  PVDF membrane

  ↓

  3 Whatman papers
- Roll the bubbles out by pressing the pack using 10 ml pipet tube
- Sprinkle some transfer buffer on the top
- Close the lid and secure it with a heavy object

- Electrophoresis:

  Volt : 250 --- Current : **102** --- Time : **1.2** --- Prog : T/V-H

  --- Keep current constant

  ▲ check 1x TBS Tween volume (**50 ml** 10x TBS + **250 µl** Tween → add dd-H_2O to total volume **500 ml**)

  ▲ prepare 5% Blotto 100 ml (**5 gm** milk in **1x TBS Tween**, total **100 ml**) stirring at least 30 mins

Protein check and blocking

- Mark membrane before removing gel

212	KDa
132	K
86	K
43.8	K
32	K
17	K
7.6	K

- Place membrane in a tray with Ponceau S., then place on shaker for 5 mins (until red bands appear)
- Wash with water and place on shaker until water is no more red
- Place membrane in a tray with 50 ml blotto
- Put in cold room shaker overnight (or 1 hour in room temperature)

Day 2

Primary antibody staining

▲ prepare 5% Blotto 100 ml (**5 gm** milk in **1x TBS tween**, total **100 ml**)

stirring at least 30 mins

- Seal two sides of hybridization bag
- Place membrane in hybridization bag
- Seal the third side (leave small space about 2-3 mm)
- Push the bubbles out with kimwipe
- Add **4 ml** blotto and **20 µl** primary antibody (VEGF-C (H-190), rabbit polyclonal antibody, 1:200)

  ** recommended dilution of VEGF-C Ab 1:200 -- 1:1000 **
- Push the bubbles out with finger and seal the last side, mix content with finger
- Put on the wheel incubator for 1 hour at room temperature

  ▲ check 1x TBS Tween volume (50 ml 10x TBS + 250 µl Tween → add dd-H_2O to total volume 500 ml)
- Wash membrane in 1x TBS tween for 4 times (10 mins each times)

Secondary antibody staining

- Put membrane in a tray with 45 ml blotto
- Add **9 µl** secondary antibody (**goat anti-rabbit HRP**) (1: 5,000),

  put tray in shaker for 1 hour at room temperature

  ▲ check 1x TBS Tween volume (50 ml 10x TBS + 250 µl Tween → add dd-H_2O to total volume 500 ml)
- Wash membrane in 1x TBS Tween for 4 times (10 mins each times)

  ▲ prepare saran wrap, kimwipe, tape, film box, film, ECL, ECL tray, 5 ml pipet tube x 2

- Light off
- Add 2 components of ECL into tray (3 ml each), mix
- Put PVDF membrane in for 1 min, reverse for another 30 sec
- Dry quickly on kimwipe
- Wrap in saran wrap (front side down)
- Tape it in film box (front side up)
- Expose film (2F, radiology dark room), fold right upper corner of film

  expose 30 sec, reverse side for 1 min

細胞株培養的方法、細胞株傳遞的方法、細胞株治療的方法(附錄4)

Maintenance media

Reagent (stock conc)	Volume needed	storage
DMEM/F12 50/50 mix (cellgro)	1 L	4°C
Bovine calf serum (BCS) (50 ml/bottle)	100 ml (2 bottle)	-20°C
Bovine Insulin (5 mg/ml)	2 ml	-20°C
TSH (10 I.U./vial)	1 vial	4°C
Fungizone (250 μg/ml)	1 ml	-20°C
Penicillin/streptomycin (10,000 U/ml)	5 ml	-20°C
L-glutamine	12.5 ml	-20°C

H5 media

Reagent (stock conc)	Volume needed	Final conc	storage
DMEM/F12 50/50 mix (cellgro)	1 L		4°C
Bovine Insulin (5 mg/ml)	2 ml	10 μg/ml	-20°C
Human transferring (10 mg/ml)	0.5 ml	5 μg/ml	-20°C
Somatostatin (10 μg/ml)	1 ml	10 ng/ml	-20°C
Glycyl-histidyl-1-lysine acetate (5 μg/ml)	0.4 ml	2 ng/ml	-20°C
Hydrocortisone (1 μg/ml)	0.36 ml	0.36 ng/ml	-20°C
L-glutamine	12.5 ml		-20°C

Cell growth protocol

-2 plate cell (count cell) in 6, 12, 96 wells with regular media

-1 change regular media to H5 media

0 Treatment day --- H5 with TSH

1

cell count :

after centrifugation

50 µl media + 50 µl trypsin blue stain

-- take 10 µl mixture to counting slide

-- blue : dead cell, clear : live cell

-- cell conc (cell/ml) = #cell /2 $*10^4$

-- alcohol wash slide

PTC-1 :250,000

FTC-133 : 350,000

FTC-236

FTC-238 : 350,000

XTC-1 : 400,000

ARO

TSH

10 I.U. (international units) per vial

2 I.U./mg protein -- 5.4 mg prot./vial

250,000 for Petri dish

cell count (cell/ml) = cell #/2 * 10^4

cell # x vol (ml) = 50

cell # 200 → 0.25 ml

cell # 100 → 0.5 ml

cell # 50 → 1 ml

cell # 25 → 2 ml

TSH concentration : (normal range : **0.5 -- 5 mU/ml**)

100 mu/ml x 10 ml = 1000 mu = 1 u

50 mu/ml x 10 ml = 500 mu = 0.5 u

10 mu/ml x 10 ml = 100 mu = 0.1 u

5 mu/ml x 10 ml = 50 mu = 0.05 u

0.5 mu/ml x 10 ml = 5 mu = 0.005u

Total 1.16 unit for 1 experiment

Choose : 0(1), 0.5(2), 5(3), 10(4), 100(5)

TSH time-course relationship

0, 10 mU/ml TSH

0, 0.5, 1, 2, 4, 8, 12, 72 hrs

TPC-1, 470,000 cells

maintenance media x 2 days → H5 media x 1 day → treatment

Time-course and dose-dependent relationship

June 28, 2002

0, 2, 4, 6, 8, 10, 12, 24 hrs

0, 10 mU/ml TSH

2, 4, 24 hrs

0, 0.1, 1, 10, 50, 100 mU/ml TSH

TPC-1, 500,000 cells

maintenance media x 2 days → H5 media x 1 day → treatment

萃取核醣核酸、測定核醣核酸濃度([redacted])

RNA isolation from cultured cells (cell line)

(TRIzol reagent method)

** When working with TRIzol reagent, use gloves and eye protection -- avoid contact with skin and clothing -- use in chemical fume hood -- avoid breathing vapor.

** assign a numerical label (i.e. Rxxx) for each sample based on the last number entered in the DNA/RNA logbook

** use aerosol resistant pipet tips

1. Harvest cells by using cell scraper or trypsin
2. Pellet cells
3. Aspirate media
4. Add 1 ml TRIzol reagent (per 5-10 million cells)
5. Resuspend pellet, keep on ice
6. Transfer to a 1.5 ml eppendorf tube

   ** or store at -80°C till next step
7. Leave (incubate) cell suspension for 5 minutes at room temperature
8. Add 0.2 ml chloroform (per 1 ml TRIzol)

   ** using 200 µl pipet tip, and change pipet tip each tube
9. Cap tubes securely
10. Vortex for 15 seconds
11. Incubate for 5 minutes at room temperature
12. Centrifuge the samples at 12,000 x for 15 minutes at 4°C, (Keep on ice)

13. Transfer the upper aqueous phase to a clean 1.5 ml eppendorf tube on ice

    ** using a P-200 pipettor (set it at 150 μl), and 200 μl pipet tip → 50 μl tip

    ** avoid disturbing the white interphase and lower phase (pink color)

14. Add 0.5 ml isopropanol (isopropyl alcohol) per 1 ml TRIzol

    ** to precipitate the RNA from the aqueous phase

    ** using P-1000 pipettor and 1000 μl pipet tip

15. mix the tube by inverting 3-4 times

16. incubate on ice for minimum of 15 minutes

    ▲ prepare agarose gel for electrophoresis

    ** alternatively : incubate at -20°C for 1 hour to overnight

17. centrifuge at 12,000 x for 10 minutes at 4°C

    ** keep on ice after centrifugation

    ** the RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube

18. remove the supernate

    ** pour directly from another side of pellet

19. add 0.5 ml 70% EtOH/DEPC (from another side of pellet slowly)

20. centrifuge at 12,000 x for 10 minutes at 4°C

    ▲ prepare 0.5 ml eppendorf tube (samples + 1 blank) for spectrophotometer

21. pipet off the 70% EtOH/DEPC

    ** can pour directly

    ** try to remove as much as possible

    ** can keep tube open for 5 minutes

22. dry the wall of eppendorf tube with a clean kimwipe

23. incubate pellet using appropriate volume of aquanase-free water for 5 minutes

    ** 25μl for small pellet and 50 μl for bigger pellet

** put aquanase-free water on the pellet directly

24. resuspend pellet with pipet

** leave on ice until following step

25. quantitate (measure RNA concentration) using spectrophotometer

** using 2.5 µl RNA and 122.5 µl dd- H_2O

** 260/280 > 1.75 is pure

** RNA conc = [dsRNA] (µg/µl) = A260 x 2

** enter concentration in RNA/DNA logbook

26. electrophoresis to check RNA intact (or degradation)

** load well using 6 µg of RNA sample and in 6x loading buffer (total 10 µl)

** if 28S and 18S are present, the RNA is intact

** using 1.5% agarose TBE gel

27. store at -80°C

** RNA isolation : 25-50 µl RNA – 2.5 µl in spectrophotometer – (1-4) µl in electrophoresis

** 1-5 µl RNA diluted to conc 1 µg/µl RNA (about 10 µl) → 1µl produce 20 µl cDNA → 2µl cDNA needed for PCR reaction

Spectrophotometer

(RNA concentration measurement)

- Turn on UV light on spectrophotometer

  ** it takes about 5-10 minutes to let machine warm up

  ** UV sign on screen will become bigger

  ** prepare "RNA only" cuvette

- Hit : program (PROG) → func → enter (ENTER) → R/S

  ** maybe it needs several R/S hits to show "insert blank"

- Until it says "insert blank", then insert blank cuvette (125 μl dd-H_2O)

  ** number on cuvette wall should "face to operator" when put in chamber

- Hit R/S

- Place 2.5 μl RNA and 122.5 μl dd- H_2O (total amount 125 μl) into cuvette

- Hit R/S again

- Repeat above two procedures

- Turn off UV

- Wash cuvette with dd- H_2O three times and ethanol two times

  ** 260/280 > 1.75 is pure

  ** RNA conc = [dsRNA] (μg/μl) = A260 x 2

Electrophoresis of RNA extraction

- Prepare gel bed by sealing ends with scotch tape

  ** be sure to fold one end of the tape

- Preparation of 1.5 % Agarose gel

  ▲ For 10x TBE buffer

  Add 0 .75 gm Agarose, 45 ml dd-H_2O for small gel (12 wells)

  Add 1.5 gm Agarose, 90 ml dd-H_2O for large gel (20 wells)

  OR

  ▲ For 20x TBE buffer

  Add 0 .75 gm Agarose, 47.5 ml dd-H_2O for small gel (12 wells)

  Add 1.5 gm Agarose, 95 ml dd-H_2O for large gel (20 wells)

  ** now we use 10x TBE buffer in our lab

- Mix

- Microwave until the agarose is completely dissolved

  ** time cook → 3:00 → start

- Mix, when cool to touch,

  ▲ For 10x TBE buffer

  Add 5ml 10x TBE buffer and 5 µl of ethidium bromide (small gel)

  Add 10 ml 10x TBE buffer and 10 µl of ethidium bromide (large gel)

  OR

  ▲ For 20x TBE buffer

  Add 2.5ml 20x TBE buffer and 5 µl of ethidium bromide (small gel)

  Add 5ml 20x TBE buffer and 10 µl of ethidium bromide (large gel)

  ** the stock Ethidium bromide concentration is 10 mg/ml stock

** the total amount for small gel is 50 ml, and for large gel is 100 ml

- let cool, then pour, making sure the comb don't touch the gel bed
- load well using 6 μg of RNA sample and in 6x loading buffer (total 10 μl)

RNA sample	A260	6 μg RNA volume needed (μl) 6/A260 x 2	6x loading buffer vol needed (μl) 10 - 6/A260 x 2
TPC-1 P16	3.0148	1.0	9.0

- run 50-100 volts for 1-1.5 hour, making sure that the loading buffer runs to a distance of 3/4 of the gel

  ** be sure the electrode is from black to red

  ** electrophoresis setting : volt, time and program at S1
- Photograph

Protein extraction

- Aspirate medium
- Wash with 3 ml PBS
- Aspirate
- Add 1 ml PBS (0.9 ml)
- Harvest cell with rubber policeman
- Transfer cell to 1.5 ml eppendorf tube
- Rinse with another 0.5 ml PBS, add to eppendorf tube, keep on ice
- Centrifugation at 900 rpm, 4°C, for 5 mins

  ** at Prof. Lin's lab

  ** lock → switch on → time adjust to 10 mins (due to timer error)
- Aspirate supernant, leave pellet
- In a new 1.5 ml eppendorf tube, mix and vortex

  ▲ 1 ml RIPA buffer (4°C)

  ▲ 1 μl aprotinin (-20°C)

  ▲ 1 μl leupeptin (-20°C)

  ▲ 10 μl PMSF (-20°C)
- Resuspend cell in 45 μl above mixture (variable volume, depend on pellet size)

  ** using 200 μl pipet tip (don't use 50 μl pipet tip)
- Shaking in eppendorf shaker for 30 mins (cold room)
- Centrifugation at max speed, 4°C, for 10 mins
- Transfer supernant to new eppendorf tube, keep in -80°C

  ** using 50 μl pipet tip then 10 μl pipet tip

  ** divided into multiple tubes (10 μl per tube)

Analysis of Protein Extraction

Preparation of protein Assay Reagent mixture

** No = sample No +8 standards + 1

- In a 50 ml test tube

  Add BCA protein assay reagent A : 1 ml x No

- Add BCA protein assay reagent B : 20 μl x No
- Mix, vortex
- Add 1 ml mixture into each 1.5 ml eppendorf tube (sample No + 8)

  ▲ adjust heater to 37°C (low switch)

- Add prepared standards and samples to the lid of eppendorf tube

- Prepare 8 standards

  ** using 0.5-10 μl pipet and 10 μl pipet tip

Conc (mg/ml, μg/μl)	BSA (2mg/ml) vol (μl)	dd-H_2O (μl)
0	0	20
0.2	2	18
0.4	4	16
0.6	6	14
0.8	8	12
1.0	10	10
1.2	12	8
0	0	20

- Prepare samples

  4 µl samples and 16 µl dd-H_2O (5 x dilution)

  ▲ check heater temperature at 37°C

- Closure of eppendorf tube carefully
- Mix by shaking up and down between two racks
- Spin down
- Incubate at 37°C heater for 30 min
- Spin down
- Spectrophotometry

  VIS → ABS → 5 → 6 → 2 →**λ**

- Load standard 0

  CALB (till reading is 0.00)

  → Read → func → 6 (it will print)

  ** using plastic cuvette

  ** triangular marker of cuvette faces to left

  ** using Pasteur pipet

- Aspirate standard 0 out

  ** using the same Pasteur pipet

  ** put plastic cuvette in situ

- Load standards in order 0.2 → 0.4 → 0.6 → 0.8 → 1.0 → 1.2

  Read → func → 6 (it will print)

- Load another standard 0

  CALB (till reading is 0.00)

  → Read → func → 6 (it will print)

- Load samples in order

  Read → func → 6 (it will print)

副甲狀腺之冰凍保存([redacted])

- centrifuge patient's serum (2K, 10 minutes)
- prepare the freezing media (4.0 ml RPMI media, 0.5 ml DMSO (dimethyl sulfoxide), 0.5 ml patient serum)
- vortex the above mixture
- transfer 1.0 ml of the freezing media to each cryovial
- cut the parathyroid tissue to 1-2 mm size pieces using a sterile blade in sterile Petri dish
- transfer 5-6 pieces of the parathyroid tissue to the cryovials
- label cryovial and transfer to -80°C freezer

Title page

Lymphangiogenic Factors, VEGF-C and VEGF-D

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Lymphangiogenic Factors, VEGF-C and VEGF-D

Vascular endothelial growth factor (VEGF) plays an essential role in angiogenesis. Numerous investigations, during the last decade, have established the critical role of VEGF as a regulator of angiogenesis.[1-3] Recently, several additional members of the VEGF gene family have been identified, including placenta growth factor (PIGF),[4] VEGF-B,[5] VEGF-C,[6] VEGF-D,[7] and VEGF-E.[8]

Lymphatic dissemination with lymph node metastasis is a major prognostic factor for most human cancers, however, the molecular mechanisms underlying lymph node metastasis are poorly understood. Until recently lymphangiogenesis has been overshadowed by the great emphasis placed on the angiogenesis.[9] This is due to the lack of identification of lymphangiogenic factors as well as the suitable markers that can distinguish blood from lymphatic vascular endothelium. This tendency is changing rapidly after the identification of the first lymphangiogenic factor, VEGF-C.

VEGF-C

Discovery. VEGF-C was first identified in the supernatant of prostatic adenocarcinoma cells PC-3 in 1996. VEGF-C is 30% identical with $VEGF_{165}$ in the region of VEGF homology domain.[6] Novel human VEGF-C gene was mapped to chromosomes 4q34, close to the human aspartylglucosaminidase gene.[10]

Expression. VEGF-C mRNA is present in multiple human tissues, with higher levels in the heart, placenta, muscle, ovary, and small intestine. In contrast, only small amounts of VEGF-C mRNA have been found in the brain, liver, or thymus.[6] VEGF-C mRNA has also been detected in about 50 percent of the unselected human malignant tumors, including lymphoma, melanoma, sarcoma, squamous cell carcinoma, and breast carcinoma.[11] The VEGF-C mRNA, by in situ hybridization analysis, is

distributed rather evenly in tumor cells. This observation differed from previous reports documenting that VEGF mRNA levels were highest in hypoxic areas of the tumors, particularly areas surrounding tumor necrosis.[11] Recent reports document that upregulation of VEGF-C occurs in many different human primary tumors, when compared to paired or unpaired normal tissues, including head and neck squamous cell carcinomas,[12, 13] esophageal squamous cell carcinomas,[14] colorectal cancers,[15] pancreatic cancers,[16] thyroid cancers,[17] and cervical cancers.[18]

Surprisingly, there is downregulation of VEGF-C in lung adenocarcinomas[19] and no difference in breast cancers,[20] when compared to unpaired normal tissue.

Regulation. VEGF-C mRNA levels are upregulated by serum and its components including platelet-derived growth factor, epidermal growth factor, transforming growth factor-β and the tumor promoter phorbol myristate 12,13-acetate.[21] Interlukin-1β, tumor necrosis factor-α, and fibroblasts have also been shown to upregulate VEGF-C mRNA expression in the human lung and umbilical vein endothelial cells.[22] These observations suggest that certain proinflammatory cytokines regulate the lymphatic vessels indirectly via VEGF-C. However, unlike VEGF-A, hypoxia, Ras oncoprotein and mutant p53 tumor suppressor genes do not appear to influence VEGF-C mRNA levels.[21]

Ligand and receptor. VEGF-C was found to be a dual ligand for VEGF receptors, VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4).[6] The specificity of VEGF-C for its two receptors is regulated by proteolytic processing.[23] VEGF-C is first synthesized as a prepropeptide of 61 kDa and a homodimer of 21 kDa is subsequently formed by proteolytic maturation. The stepwise proteolytic processing of VEGF-C generates several VEGF-C forms with increased activity towards VEGFR-3, but only the fully processed VEGF-C can activate VEGFR-2. However, the respective roles of VEGFR-2 and VEGFR-3 in mediating the biological effects of VEGF-C are not

completely understood. The VEGFR-3 is a receptor tyrosine kinase that is similar to the two VEGF receptors, VEGFR-1 (Flt-1) and VEGFR-2, in structure, but VEGFR-3 does not bind with VEGF, PIGF or VEGF-B. Moreover, the expression of VEGFR-3 becomes highly restricted to the lymphatic endothelium in adult tissues.[24] Therefore, VEGF-C is considered to be a lymphangiogenic factor.

Lymphangiogenesis. Further investigations have established the lymphangiogenic role of VEGF-C. Jeltsch et al. showed that transgenic overexpression of VEGF-C in keratocytes using keratin 14 promoter results in lymphatic, but not vascular, endothelial proliferation and vessel enlargement in the mouse skin.[25] Another investigation in the differentiated avian chorioallantoic membrane documented that recombinant VEGF-C protein induces lymphangiogenesis, but has only a mild angiogenic response.[26] Signaling via VEGFR-3 alone has been shown to be sufficient for inducing lymphatic hyperplasia. Transgenic mice (VEGF-C156S) overexpressing a mutant form of VEGF-C, which binds and activates VEGFR-3, but has lost its capacity to bind VEGFR-2, is able to induce a similar phenotype.[27]

Angiogenesis. VEGF-C, however, binds to VEGFR-2 expressed in blood vessel endothelia and stimulates the migration of bovine capillary endothelial cells in a collagen gel.[6] The potential for VEGF-C to promote angiogenesis in vivo was tested in a rabbit ischemic hindlimb model and demonstrated that constitutive expression of VEGF-C in adult animals promoted angiogenesis.[28] Another investigation documented that ectopic application of recombinant VEGF-C had potent angiogenic effects and stimulated neovascularization of limbal vessels in the mouse cornea.[29] Thus, VEGF-C has a dual biological role in both lymphangiogenesis and angiogenesis.

VEGF-D

VEGF-D is the most recently discovered member of the VEGF family.[7, 30] Human VEGF-D is located on chromosome Xp22.31 and VEGF-D mRNA is expressed predominantly in the heart, lung, skeletal muscle, colon, and small intestine.[30] It shares 61% sequence homology with VEGF-C in the region of VEGF domain. VEGF-D and VEGF-C are also similar from a functional point of view, because receptor-binding studies demonstrate that VEGF-D exhibited similar receptor specificities to those of VEGF-C. VEGF-D binds and activates both the VEGFR-2 and VEGFR-3. VEGF-D is proteolytically processed similar to VEGF-C, and the proteolytic processing also appears to regulate its biological activity and receptor specificity.[31]

Markers of lymphatic endothelium

Another major advance in the field of lymphangiogenesis has been the discovery of specific lymphatic endothelial markers. Over the past few years, several molecules expressed specifically in the lymphatic endothelial cells have been characterized. VEGFR-3 was the first molecule found to be expressed in the lymphatic endothelium,[32] and further studies revealed that it is also expressed in blood vessels within certain tumors.[33, 34] Podoplanin, an integral membrane mucoprotein first found in glomerular epithelial cells, is also expressed in the lymphatic endothelium.[35] Prox-1, a homeobox gene, is a specific and required regulator of early lymphatic development.[36] Prox-1 protein is present in the nuclei of lymphatic endothelial cells but absent from the blood vascular endothelium. Lymphatic vessel endothelial hyaluronan receptor-1, LYVE-1, is a lymphatic endothelial receptor for the extracellular matrix glycosaminoglycan hyaluronan. It, however, is not completely specific for the lymphatic endothelial cells.[37, 38] More information needs to be

obtained to clarify the specificity of these markers in lymphatic vessels during disease processes. Therefore, until the specificity of these markers in lymphatic vessels during tumor progression and metastasis has been clarified, multiple markers should be used in determining the importance of lymphatic vessels density, tumor growth, metastasis, and prognosis.[39]

Human disorders associated with lymphangiogenesis

Lymphangioma (VEGF-C/VEGFR-3 upregulation). Abnormal development or function of the lymphatic endothelial cells occasionally results in tumors or malformations of the lymphatic vessels, such as lymphangiomas or lymphangiectasias. There are minimal data regarding the VEGF family members in the tumors of lymphatic vessels. Coexpression of VEGF-C and VEGFR-3 mRNA, however, has been found in human lymphangioma.[40]

Lymphedema (VEFR-3 missense mutation). Human hereditary lymphedema, characterized by a chronic and disfiguring swelling of the extremities, was reported to be associated with heterozygous missense mutations of VEGFR-3 which inactivated the tyrosine kinase domain and subsequently led to insufficient VEGFR-3 signaling.[41, 42]

VEGF-C, lymphangiogenesis, and lymph node metastasis

The hematogenous metastasis of human cancer is known to involve invasion of intratumoral blood vessels generated by the process of tumor angiogenesis. Microvascular density has been used as a measure of tumor angiogenesis, and its correlation to tumor growth, metastasis, and prognosis. Relatively little is known, however, about the mechanisms of metastatic spread via the lymphatics. It is uncertain whether tumors also induce lymphangiogenesis by secreting VEGF-C or

simply invade existing peritumoral vessels. If lymphangiogenesis takes place during cancer progression, cancers with active lymphangiogenesis could spread from the primary tumor via the lymphatic system and thus make complete removal more difficult. There have been a number of investigations that have tried to clarify the relationship between VEGF-C expression, tumor lymphangiogenesis, and the formation of regional lymph node metastasis.

In human cancers. Many clinical investigations have documented a positive correlation between VEGF-C levels in primary tumors and the presence of lymph node metastases. These include thyroid,[17, 43] head and neck squamous cell carcinomas,[12] lung,[44] esophageal squamous cell carcinomas,[14] gastric,[45-47] pancreatic,[16] colorectal,[48] prostate,[49] and cervical cancers.[50, 51] The association of VEGF-C with lymphatic metastasis, however, has not been identified in all tumor types. Several investigations have reported no difference in VEGF-C expression relative to lymph node status in lung carcinomas,[19] breast cancers,[20] malignant mesothelioma,[52] neuroblastomas,[53] and colorectal cancers.[15] In addition, some studies have reported a positive correlation of lymph node status to VEGF-A in lung[54] and colorectal cancers.[15, 55] Others have reported similar relationship to VEGF-D in colorectal cancers,[56] and to VEGF-C/VEGF-D ratio in lung cancers.[19] A positive correlation between the VEGF-C expression in the primary tumors and a poorer prognosis has been reported in cervical[50, 51] and gastric cancers.[45, 47] However, other studies in colorectal[48] and pancreatic cancer[16] failed to identify such correlation. The reasons for the differences identified above are not clearly known but may relate to sample selection or preparation, to different techniques for quantification, or because different mechanisms are involved in lymphatic recruitment and growth in different tumors.

A number of questions have been raised by these observations. First, although

VEGF-C expression of primary tumors in some cases correlated with nodal metastasis, its direct effect on lymph vessel proliferation remains to be demonstrated. In human cancers, however, the phenomenon of tumor lymphangiogenesis needs more investigations. The study from Beasley et al. showed that the high intratumoral lymph vessel density was associated with neck node metastases in oropharyngeal carcinomas, however, VEGF-C mRNA levels in tumor tissue had no obvious correlation with intratumoral lymphatics.[13] The mechanisms by which increased expression of VEGF-C in primary tumors results in an increase in lymph node metastases needs further clarification.

In animal model. Skobe et al.[57] demonstrated that intratumoral lymphangiogenesis and enlargement of peritumoral lymphatic vessels occurred within human VEGF-C overexpressing MDA-MB-435 breast cancers that were orthotopically transplanted onto nude mice. Increased density of lymphatic vessels within the tumor was associated with a significantly increased incidence of metastasis to regional lymph nodes. Mattila et al. showed that there was a significantly increased incidence of regional lymph node metastasis in nude mice bearing orthotopic tumors formed by MCF-7-VEGF-C cells than MCF-7-Mock cells. The density of intratumoral and peritumoral lymphatic vessels was increased in tumors derived from MCF-7-VEGF-C cells but not MCF-7-Mock cells.[58] Mandriota et al. showed that Rip VEGF-C transgenic mice, in which VEGF-C expression driven by the rat insulin promoter (Rip) was targeted to pancreatic β-cell tumors, developed lymphangiogenesis and lymph node metastases.[59] Overexpression of VEGF-D in orthotopically transplanted 293EBNA fibrosarcoma also promotes tumor lymphangiogenesis and subsequent lymph node metastasis in mice.[60] In a study by Padera et al.,[61] overexpression of VEGF-C in orthotopically transplanted T-241 murine fibrosarcomas and B16-F10 murine melanomas correlated with lymphatic metastasis. However, these tumors

contained no functional lymphatics, as assessed by four independent functional assays and immunohistochemical staining, suggesting that functional lymphatics in the tumor margin alone are sufficient for lymphatic metastasis.

Implications for the human disease therapy

Therapeutic lymphangiogenesis. The pathogenesis and possible treatment for lymphedema has recently been studied using the Chy mouse model.[62] By using virus-mediated VEGF-C gene therapy, it was possible to generate new functional lymphatic vessels in mice with lymphedema. The results suggest that growth factor gene therapy is applicable to human lymphedema and provides a paradigm for other diseases associated with mutant receptors.

Inhibition of lymphangiogenesis (antimetastatic therapy). The inhibition of VEGF activity by specific monoclonal antibodies has been reported to reduce the growth of experimental tumors and their blood vessel density.[63] The inhibition of lymphangiogenesis represents a new target for the development of anti-cancer therapies. Inhibition of VEGFR-3 function might be a therapeutic approach in human tumors that express high levels of VEGF-C and VEGF-D and have more lymphatic metastases such as in papillary thyroid cancers. Accordingly, VEGF-C together with VEGF-D could be molecular targets for the prevention of lymph node metastases. Interesting data have recently been published showing inhibition of lymphangiogenesis by soluble VEGFR-3. Makinen et al. showed that a soluble form of VEGFR-3 was a potent inhibitor of VEGF-C/VEGF-D signaling, and when expressed in the skin of transgenic mice, it inhibited fetal lymphangiogenesis and induced regression of already formed lymphatic vessels.[64] Another investigation in VEGF-C overexpressing MCF-7 breast carcinoma cells showed that VEGF-C overexpression was associated with lymphangiogenesis and intralymphatic growth of

tumor cells, and that VEGF-C induced lymphangiogenesis could be inhibited by a circulating soluble VEGFR-3 fusion protein when tumors were transfected with expression plasmids coding for full-length human VEGF-C or a soluble VEGFR-3 fusion protein (VEGFR-3-Ig).[65] Lymphangiogenesis was also inhibited to some extent in avian CAM assay when A375 melanoma cells were transfected with cDNA encoding soluble VEGFR-3.[66]

Further approaches to the inhibition of lymphangiogenesis and lymphatic metastasis will include direct blocking of the function of VEGF-C and VEGF-D and manipulation of their receptors.

Caution. Inhibition of lymphangiogenesis in transgenic mice expressing soluble VEGFR-3 was reported to produce severe lymphedema.[64] This study suggests that systemic targeting of VEGF-C (and other lymphangiogenic molecules) could increase the risk of lymphedema in patients. Similarly, any trial designed to assess the efficacy of VEGF-C treatment for reversing lymphedema in patients who have cancer must take into account the possibility that VEGF-C induced lymphangiogenesis could enhance lymphatic metastasis.[67]

Conclusion

The discovery of lymphangiogenic factors, VEGF-C and VEGF-D, and markers of lymphatic endothelium provides new methods to determine the factors involved in lymphangiogenesis. The role of VEGF-C and VEGF-D and their interaction with each other and other factors in tumor lymphangiogenesis and lymph node metastasis needs to be further clarified. Some investigations but not all document a positive relationship of VEGF-C and VEGF-D expression and lymph node metastases in both human and animal cancer models. The early results in animal models about therapeutic lymphangiogenesis for treatment of lymphedema and lymphangiogenesis

inhibition as an antimetastatic therapy are encouraging. Prior to clinical application, more research need to be done.

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Title page

Expression of Vascular Endothelial Growth Factor-C in Benign and Malignant Thyroid Tumors

Abbreviated title: VEGF-C expression in thyroid tumors

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Abstract

In contrast to vascular endothelial growth factor (VEGF), which stimulates angiogenesis, VEGF-C is thought to stimulate lymphangiogenesis. The role of VEGF-C in thyroid cancer pathogenesis has not been clarified. One might expect a different pattern of VEGF-C expression in the various types of thyroid cancer because of their different means of metastases. In this investigation, we determined whether the differential expression of VEGF-C might explain the different propensity to lymph node metastasis in thyroid cancers. One hundred and eleven normal and neoplastic thyroid tissues were analyzed by real-time quantitative PCR. Papillary thyroid cancers had a higher VEGF-C expression than other thyroid malignancies ($P < 0.0005$ ANOVA). Among the normal thyroid tissues from patients with malignant or benign thyroid diseases, there was no significant difference in VEGF-C expression. Paired comparison of VEGF-C expression between thyroid cancers and normal thyroid tissues from the same patients showed a significant increase of VEGF-C expression in papillary thyroid cancer (1.10 ± 0.41 vs. 0.70 ± 0.13; $P = 0.001$) and a significant decrease of VEGF-C expression in medullary thyroid cancer (0.11 ± 0.13 vs. 0.78 ± 0.29; $P = 0.001$). In contrast, there was no significant difference of VEGF-C expression between cancer and normal tissues in other types of thyroid cancer. In summary, VEGF-C expression is increased in papillary thyroid cancer compared with paired normal thyroid tissues, but not in other thyroid cancers that are also prone to lymph node metastasis. The lymphangiogenic role of VEGF-C in thyroid cancers therefore appears to be complex and other factors are likely to be also involved.

Introduction

Lymph node metastasis is common in most cancers but the molecular mechanisms underlying lymph node metastasis are poorly understood. In contrast to vascular endothelial growth factor (VEGF), which stimulates angiogenesis, VEGF-C is thought to stimulate lymphangiogenesis. VEGF-C was found in the supernatant of prostate cancer cells (PC-3) in 1996 (1). It is 30% identical with human splice variant VEGF165. VEGF-C is expressed in multiple human tissues, predominantly in the heart, placenta, muscle, ovary, and small intestine. VEGF-C binds to the VEGF receptor, VEGFR-3 (Flt-4) and the expression of VEGFR-3 is highly restricted to the lymphatic endothelium in adult tissues (2). Transgenic overexpression of VEGF-C in keratocytes caused lymphatic, but not vascular, endothelial proliferation and vessel enlargement in the mouse skin (3). Recombinant VEGF-C protein induced significant lymphangiogenesis, but only mild angiogenesis, in avian chorioallantoic membranes (4). Several studies have shown that cancers that metastasize to lymph nodes usually express more VEGF-C (5-17), but this correlation is not universal (18-22). VEGF-C expression is upregulated in many (5, 7, 9, 13, 22-24), but not in all human cancers (18, 19).

The role of VEGF-C in thyroid cancer pathogenesis has not been completely clarified. Different thyroid cancers have a different propensity for lymph node metastasis. For example, papillary thyroid cancer tends to metastasize to regional lymph nodes, whereas follicular thyroid cancer usually metastasizes by a hematogenous rather than by a lymphatic route. Other less common thyroid cancers, such as Hürthle cell cancers, medullary cancer, and anaplastic cancer also frequently metastasize to lymph nodes. In this investigation, we determined whether VEGF-C expression differed in various thyroid cancers that metastasize via a lymphatic or

hematogenous route. We hypothesized that thyroid cancers that metastasize to lymph nodes express more VEGF-C than those that do not.

Materials and Methods

Tissue samples

One hundred and eleven thyroid and lymph node tissues were obtained from patients who underwent thyroid operations at the University of California Mount Zion Medical Center, San Francisco (UCSF). The protocol was approved by the human research committee of UCSF. Tissues were obtained in the operating room after removal and immediately frozen in liquid nitrogen, and kept at -80 °C until analysis. Tumors were histologically classified according to the World Health Organization (WHO) recommendations. The thyroid tissues included 15 papillary cancers, 8 follicular cancers, 6 Hürthle cell cancers, 11 medullary cancers, 3 anaplastic cancers, 6 follicular adenomas, 5 Hürthle cell adenomas, 4 Graves' disease and 48 corresponding paired normal thyroid tissues in most of these patients when available (15 papillary cancers, 6 follicular cancers, 6 Hürthle cell cancers, 7 medullary cancers, 3 anaplastic cancers, 6 follicular adenomas, 5 Hürthle cell adenomas). Tissues from metastatic lymph nodes were also obtained from 5 patients with papillary cancer.

RNA extraction and cDNA synthesis

Total RNA was extracted from tissue specimens using the TRIzol method (Gibco BRL, Gaithersburg, MD) according to the manufacture's suggested protocol. Concentration and purity was determined using a Beckman spectrophotometer. The quality of RNA was checked by the electrophoresis of 3 μg samples in a 1.5 % agarose gel, staining with ethidium bromide. The 28S and 18S rRNA bands were

examined on a UV transilluminator. No significant degradation was observed in any RNA samples.

Total RNA (250 ng) was reverse transcribed in a total reaction volume of 100 µl containing 250 U Maloney murine leukemia virus reverse transcriptase (Gibco BRL), 40 U ribonuclease inhibitor (Roche, Nutley, NJ), 1mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP) (Amersham Pharmacia Biotech Inc., Arlington Heights, IL), 7.5 mM $MgCl_2$, 5 mM random hexamer (Invitrogen, Carlsbad, CA), and 1X PCR buffer II (Applied Biosystems, Foster City, CA). Complementary DNA was synthesized using a thermal cycler (Amplitron II Thermolyne, Barnstead / Thermolyne, IA) by incubating at 25 °C for 10 minutes, 48 °C for 40 minutes, and 95 °C for five minutes.

Real-time quantitative PCR

Oligonucleotide primers and Taqman probes were designed to be intron spanning using Primer Express software (Applied Biosystems) and purchased from Biosearch Technology (Novato, CA). VEGF-C specific primers were designed to amplify a 93 bp product. Human beta-glucuronidase (GUS) gene was used as an endogenous control to normalize the expression of VEGF-C (25). The sequences of the primers and probes of VEGF-C and GUS genes are shown at Table 1. Optimal PCR conditions and PCR efficiency were determined empirically and were > 90% efficient.

Quantitative real time PCR was then performed using a 96-well optic tray on the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). A total reaction volume of 50 µl contained 1X TaqMan Buffer A (Applied Biosystems), 5.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (dATP,

dCTP, dGTP, and dTTP), 1.25 U AmpliTaq Gold (Applied Biosystems), 500 nM of each primer, 200 nM probe and 10 μl cDNA (described above, equivalent to a cDNA amount from 25 ng of initial total RNA). The PCR thermal cycle condition was setup at 95 °C for 12 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

The mRNA content of each target gene was simultaneously determined in the 96-well. Each sample was run as a triplicate. The negative controls lacking template RNA were included in each experiment. Data collection and analysis was performed with SDS v1.7 software (Applied Biosystems). Data was then exported and further analyzed in Excel (Microsoft, Redmond WA).

The comparative Ct method

The comparative Ct method was used as previously described (26). Briefly, the difference in cycle threshold (Ct, the number of PCR cycles required for the FAM (i.e., 6-carboxyfluorescein) emission intensities to exceed a threshold) between the VEGF-C and GUS genes was calculated and designated as ΔCt. VEGF-C mRNA expression, relative to GUS, is calculated according to the following formula: $2^{-\Delta Ct}$. These calculations are valid since the measured PCR efficiencies are very close to 100%.

Statistical analysis

All data are expressed as mean ± SEM. Differences among multiple groups were analyzed using analysis of variance (ANOVA). The Tukey HSD test was used as the post-hoc test. Paired analysis was performed by paired Student's *t* test (SPSS, version

11.0; SPSS Inc, Chicago, IL). A P value < 0.05 was considered statistically significant.

Results

VEGF-C mRNA expression (Tables 2-4)

Papillary thyroid cancers had a higher VEGF-C expression than other thyroid malignancies ($P < 0.0005$ ANOVA) (Table 2). However, there was no significant difference between all malignant thyroid tissues and all benign thyroid tissues ($P = 0.767$). Among the normal thyroid tissues from patients with malignant or benign thyroid diseases, there was no significant difference in VEGF-C expression (Table 3, 4). There was no difference in VEGF-C expression between patients younger than or older than 45 years of age for the entire group of malignant neoplasms (0.70 ± 0.53 *vs.* 0.54 ± 0.49; $P = 0.313$) or for the papillary thyroid cancers (1.13 ± 0.34 *vs.* 1.00 ± 0.61; $P = 0.603$).

VEGF-C expression in paired samples (Tables 3-6)

Samples from both malignant and normal thyroid tissue were available in 48 patients. In these paired-sample analyses, the normal thyroid tissue from the same patient served as a control because VEGF-C expression may be regulated by various factors, such as epidermal growth factor, that differ among the patients.

Paired comparison of VEGF-C expression between thyroid cancers and normal thyroid tissues from the same patients showed an increase of VEGF-C expression in papillary thyroid cancers (1.10 ± 0.41 *vs.* 0.70 ± 0.13; $P = 0.001$) (Fig. 1A) and a decrease of VEGF-C expression in medullary thyroid cancers (0.11 ± 0.13 *vs.* 0.78 ± 0.29; $P = 0.001$) (Fig. 1B) (Table 3). In contrast, there was no difference of VEGF-C expression between cancer and normal tissues in other types of thyroid cancer (Figs. 1C, 1D, 1E).

Paired comparison of VEGF-C expression between benign thyroid tumors and normal thyroid tissues from the same patients showed a significant decrease of VEGF-C expression in Hürthle cell adenoma (0.52 ± 0.34 *vs.* 1.19 ± 0.33; P = 0.026) (Fig. 2B) but not in follicular adenoma (Fig. 2A) (Table 4).

To determine whether different subgroups of patients with papillary thyroid cancer have different VEGF-C expression, we analyzed those with clinically positive cervical lymph node metastases, clinically negative cervical lymph node metastases and follicular variants of papillay cancer (Table 5). There was a significant increase of VEGF-C expression in papillary thyroid cancer tissues from the node positive group (1.25 ± 0.47 *vs.* 0.65 ± 0.15; P = 0.024) (Fig. 3A) and the node negative groups (1.16 ± 0.44 *vs.* 0.67 ± 0.10; P = 0.027) (Fig. 3B). However, there was no significant difference in VEGF-C expression in a subgroup of patients with follicular variants of papillary thyroid cancer (0.81 ± 0.14 *vs.* 0.79 ± 0.12; P = 0.659) (Fig. 3C).

To determine whether VEGF-C expression differed between primary papillary thyroid cancers, metastatic lymph nodes, and normal thyroid tissues, we analyzed these three tissues from the 5 patients who presented with clinically positive cervical lymph node metastases (Table 6). VEGF-C expression in the primary thyroid cancers was higher compared to either VEGF-C expression in the metastatic lymph nodes or in the normal thyroid tissues from the same patient (cancer 1.25 ± 0.47 *vs.* normal 0.65 ± 0.15 *vs.* lymph node 0.43 ± 0.20; P = 0.003) (Fig. 4).

Discussion

Numerous investigations have previously documented a significant correlation between VEGF-C expression in primary cancer tissues and the occurrence of lymph node metastases, in thyroid cancers (5, 6), head and neck squamous cell carcinomas (7), lung (8), esophageal (9), gastric (10-12), pancreatic (13), colorectal (14), prostate (15), and cervical cancers (16, 17). However, this association of VEGF-C with lymphatic metastasis is not universally observed in all investigations. In some studies in lung cancers (18), breast cancers (19), mesothelioma (20), neuroblastomas (21), and colorectal cancers (22), there were no direct correlation between VEGF-C expression in primary cancers and lymph node metastases. The reason for the differences is not clear but may relate to sample preparation, different experimental techniques, or the differences in tumor histology.

Theoretically, one would expect higher VEGF-C expression in papillary, Hürthle cell, medullary, and anaplastic thyroid cancers than in follicular thyroid cancers since these cancers metastasize to regional lymph nodes. In our investigation, we found higher VEGF-C expression in papillary thyroid cancers. Surprisingly, however, we found a lower expression of VEGF-C in Hürthle cell, anaplastic, and medullary thyroid cancers. What we found is in part similar to one previous report showing a significantly higher VEGF-C gene expression in papillary than in follicular thyroid cancers (27). It is, however, contrary to another study (5) that reported a generalized upregulation of VEGF-C mRNA in papillary thyroid cancers (76%), undifferentiated thyroid cancers (86%), and medullary thyroid cancers (83%), when compared with unpaired normal thyroid tissues.

We also found no significant difference in VEGF-C expression in papillary thyroid cancers from patients who had or did not have cervical lymph node metastases, although both groups showed upregulation of VEGF-C expression in the thyroid cancer tissues when compared to paired normal thyroid tissues from the same patients.

One previous study reported an upregulation of VEGF-C mRNA levels in metastatic lymph nodes in patients with papillary thyroid cancers (92%) and medullary cancers (100%) compared with unpaired normal thyroid tissues (5). In contrast, when examining the VEGF-C expression of metastatic lymph nodes in 5 papillary thyroid cancer patients with clinically positive cervical lymph node metastases, we found that the VEGF-C expression in either metastatic lymph nodes or paired normal thyroid tissues was significantly lower than that found in the paired primary thyroid cancers from the same patients.

There have been conflicting reports about the expression of VEGF-C in human primary (not lymph node) cancers. VEGF-C expression was found to be increased in head and neck squamous cell carcinomas (7, 23), esophageal squamous cell carcinomas (9), colorectal cancers (22), pancreatic cancers (13), thyroid cancers (5), and cervical cancers (24). The expression was found to decrease in lung adenocarcinoma (18) and showed no difference in breast cancer (19).

One of the reasons for the conflicting results may be because non-paired normal tissue was used for the comparison in these studies. Paired-sample analysis, using the tumor and normal thyroid tissues from the same patients, controls for factors that regulate VEGF-C expression that may differ from patient to patient. VEGF-C mRNA expression is upregulated by serum and various growth factors, such as platelet-derived growth factor, epidermal growth factor, transforming growth factor-β and the tumor promoter phorbol myristate 12,13-acetate (28). Interlukin-1β and tumor

necrosis factor-α also upregulate VEGF-C mRNA expression in human lung fibroblasts and umbilical vein endothelial cells (29). In contrast, in our investigation, TSH, which stimulates the expression of many genes in thyroid cells, did not stimulate VEGF-C mRNA expression in thyroid cancer cell lines (unpublished data).

Interestingly, although we found increased VEGF-C expression in papillary cancers and decreased VEGF-C expression in medullary cancers, we found no differences in cancers and normal tissues from Hürthle cell and anaplastic thyroid cancers (although a tendency to be lower in cancers). It is possible that the lower expression of VEGF-C in Hürthle cell and anaplastic thyroid cancers might become statistically significant if more samples are studied. Taken together our results demonstrate that lymphangiogenesis is a complex process and the expression of VEGF-C is not the only mechanism in lymphangiogenesis and lymph node metastasis. Other factors are likely to be involved.

As predicted, follicular cancers and adenomas did not have an increased VEGF-C expression. Interestingly, the few follicular variants of papillary thyroid cancer that we studied, also showed no increase in contrast to other types of papillary thyroid cancer.

Inhibiting VEGF activity using monoclonal antibodies can reduce the growth and angiogenesis in cancers (30). Lymphangiogenesis may similarly be a new target for anti-cancer therapy. Inhibiting VEGF-C activity in cancers that express high levels of VEGF-C may reduce lymph node metastasis. For example, one might speculate that papillary thyroid cancer, but not the Hürthle cell, anaplastic or medullary thyroid cancer, would be a good candidate for such treatment.

In summary, VEGF-C expression is increased in papillary thyroid cancer compared with paired normal thyroid tissues, but not in other thyroid cancers that are also prone to lymph node metastasis. Therefore, the lymphangiogenic role of

VEGF-C in thyroid cancers is complex and other factors are likely to be also involved.

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TABLE 1. Primer and probe sequences used in the real-time quantitative RT-PCR

Gene	Primers (5'-3')	Probe (5'FAM-3'TAMRA)
VEGF-C	Forward: TTCATTCCATTATTAGACGTTCCCT Reverse: GATTATTCCACATGTAATTGGTGGG	CCAGCAACACTACCACAG TGTCAGGCA
GUS	Forward: CTCATTTGGAATTTTGCCGATT Reverse: CCGAGTGAAGATCCCCTTTTTA	TGAACAGTCACCGACGAG AGTGCTGG

VEGF-C, vascular endothelial growth factor-C; GUS, human beta-glucuronidase.

TABLE 2. VEGF-C expression, relative to GUS expression, in malignant and benign thyroid tissues

Pathology	N	Ct value		VEGF-C expression
		GUS	VEGF-C	
PTC	15	24.75 ± 1.16	24.70 ± 1.09	1.10 ± 0.41
FTC	8	24.45 ± 0.46	25.14 ± 0.69	0.67 ± 0.27
HTC	6	25.58 ± 3.16	27.39 ± 3.94	0.45 ± 0.46
MTC	11	25.47 ± 1.27	29.76 ± 2.50	0.09 ± 0.10
ATC	3	25.92 ± 0.57	27.53 ± 0.67	0.41 ± 0.31
FA	6	24.57 ± 0.60	24.99 ± 0.63	0.80 ± 0.32
HA	5	23.81 ± 1.11	25.04 ± 0.60	0.52 ± 0.34
Graves'	4	23.53 ± 1.05	24.28 ± 1.47	0.63 ± 0.25
normal	48	24.96 ± 0.87	25.33 ± 0.99	0.85 ± 0.36

PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; HTC, Hürthle cell thyroid cancer; MTC, medullary thyroid cancer; ATC, anaplastic thyroid cancer; FA, follicular adenoma; HA, Hürthle cell adenoma.

N is the number of samples in each group; Ct, the number of PCR cycles required for the FAM emission intensities to exceed a threshold.

TABLE 3. Paired comparison of VEGF-C expression, relative to GUS expression, between malignant thyroid tumors and normal thyroid tissues from the same patients

Pathology	N	VEGF-C expression		P
		Normal	Tumor	
PTC	15	0.70 ± 0.13	1.10 ± 0.41	0.001*
FTC	6	0.65 ± 0.13	0.65 ± 0.23	0.987
HTC	6	1.03 ± 0.69	0.45 ± 0.46	0.089
MTC	7	0.78 ± 0.29	0.11 ± 0.13	0.001*
ATC	3	1.24 ± 0.44	0.41 ± 0.31	0.062

* $P < 0.05$ by paired Student's *t* test.

PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; HTC, Hürthle cell thyroid cancer; MTC, medullary thyroid cancer; ATC, anaplastic thyroid cancer.

N is the number of samples in each group.

TABLE 4. Paired comparison of VEGF-C expression, relative to GUS expression, between benign thyroid tumors and normal thyroid tissues from the same patients

Pathology	N	VEGF-C expression		P
		Normal	Tumor	
FA	6	0.87 ± 0.24	0.80 ± 0.32	0.573
HA	5	1.19 ± 0.33	0.52 ± 0.34	0.026*

* $P < 0.05$ by paired Student's *t* test.

FA, follicular adenoma; HA, Hürthle cell adenoma.

N is the number of samples in each group.

TABLE 5. Paired comparison of VEGF-C expression, relative to GUS expression, in different subgroups of patients with papillary thyroid cancer

PTC	N	VEGF-C expression		P
		Normal	Tumor	
LN meta (+)	5	0.65 ± 0.15	1.25 ± 0.47	0.024*
LN meta (-)	6	0.67 ± 0.10	1.16 ± 0.44	0.027*
Follicular variant	4	0.79 ± 0.12	0.81 ± 0.14	0.659

* $P < 0.05$ by paired Student's *t* test.

PTC, papillary thyroid cancer; LN meta (+), positive cervical lymph node metastasis; LN meta (-), negative cervical lymph node metastasis; Tumor, thyroid cancer tissue from the thyroid; Normal, normal thyroid tissue from the same patient.

N is the number of samples in each group.

TABLE 6. Comparison of VEGF-C expression, relative to GUS expression, among papillary thyroid cancers tissues, metastatic lymph nodes, and normal thyroid tissues from the same patients

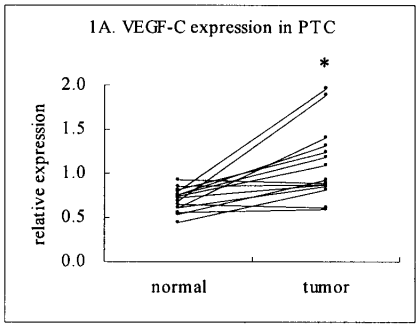
PTC	N	VEGF-C expression			P
		Tumor	Normal	Lymph node	
	5	1.25 ± 0.47	0.65 ± 0.15	0.43 ± 0.20	0.003*

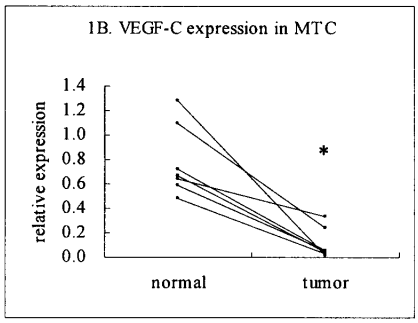
* $P < 0.05$ by ANOVA.

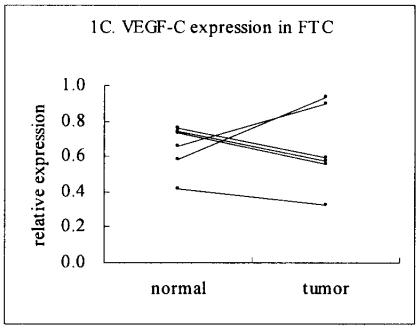
PTC, papillary thyroid cancer; Tumor, thyroid cancer tissue from the thyroid; Normal, normal thyroid tissue; Lymph node, thyroid cancer tissue from metastatic lymph node.

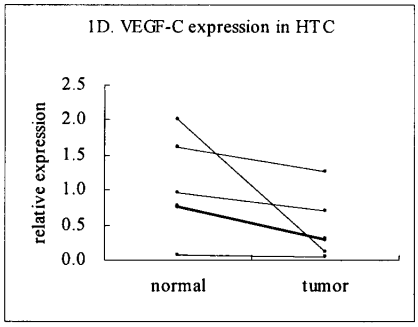
N is the number of samples in each group.

FIG. 1.


1A. VEGF-C expression in PTC
relative expression
2.0
1.5
1.0
0.5
0.0
*
normal
tumor


1B. VEGF-C expression in MTC
relative expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
normal
tumor


1C. VEGF-C expression in FTC
relative expression
1.0
0.8
0.6
0.4
0.2
0.0
normal
tumor


1D. VEGF-C expression in HTC
relative expression
2.5
2.0
1.5
1.0
0.5
0.0
normal
tumor

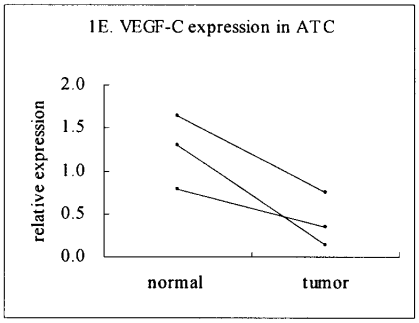

1E. VEGF-C expression in ATC
relative expression
2.0
1.5
1.0
0.5
0.0
normal
tumor

FIG. 2.

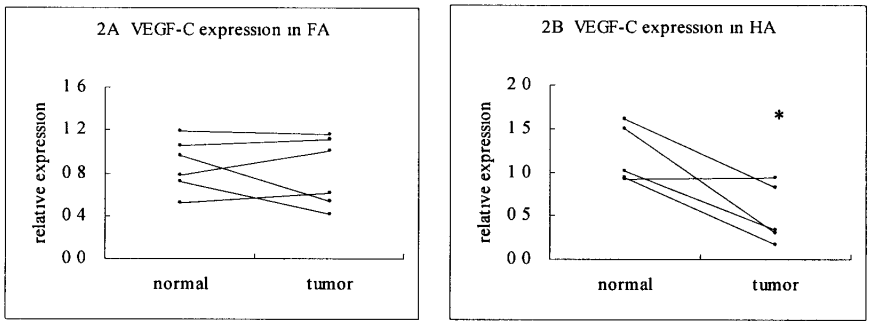
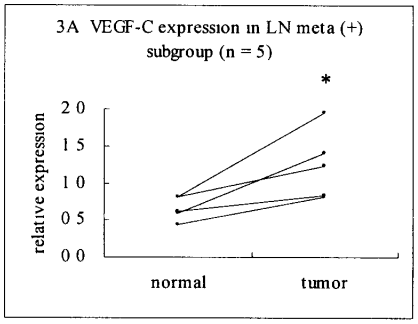
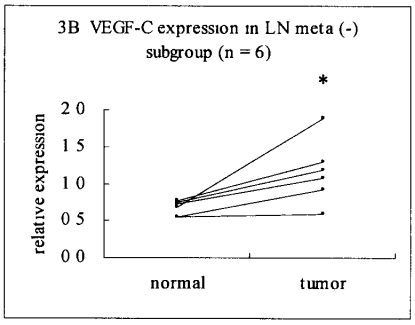

2A VEGF-C expression in FA
relative expression
1 6
1 2
0 8
0 4
0 0
normal
tumor
2B VEGF-C expression in HA
relative expression
2 0
1 5
1 0
0 5
0 0
*
normal
tumor

FIG. 3.


3A VEGF-C expression in LN meta (+) subgroup (n = 5)
*
relative expression
2 0
1 5
1 0
0 5
0 0
normal
tumor


3B VEGF-C expression in LN meta (-) subgroup (n = 6)
*
relative expression
2 0
1 5
1 0
0 5
0 0
normal
tumor

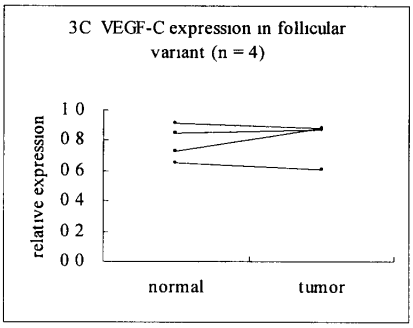
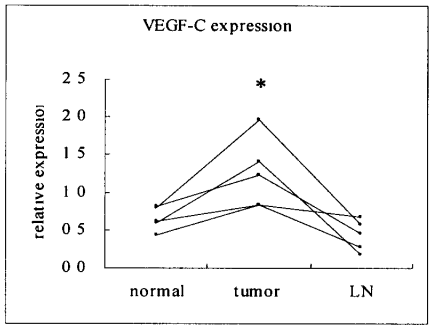

3C VEGF-C expression in follicular variant (n = 4)
relative expression
1 0
0 8
0 6
0 4
0 2
0 0
normal
tumor

FIG. 4.


VEGF-C expression
relative expression
2 5
2 0
1 5
1 0
0 5
0 0
*
normal
tumor
LN

Legends

FIG. 1. Paired comparison of VEGF-C expression, relative to GUS expression, between malignant thyroid tumors and normal thyroid tissues from the same patients (*P < 0.05, paired Student's *t* test). PTC, papillary thyroid cancer (n = 15); FTC, follicular thyroid cancer (n = 6); HTC, Hürthle cell thyroid cancer (n = 6); MTC, medullary thyroid cancer (n = 7); ATC, anaplastic thyroid cancer (n = 3).

FIG. 2. Paired comparison of VEGF-C expression, relative to GUS expression, between benign thyroid tumors and normal thyroid tissues from the same patients (*P < 0.05, paired Student's *t* test). FA, follicular adenoma (n = 6); HA, Hürthle cell adenoma (n = 5).

FIG. 3. Paired comparison of VEGF-C expression, relative to GUS expression, in different subgroups of patients with papillary thyroid cancer (*P < 0.05, paired Student's *t* test). PTC, papillary thyroid cancer; LN meta (+), positive cervical lymph node metastasis; LN meta (-), negative cervical lymph node metastasis; Tumor, thyroid cancer tissue from the thyroid; Normal, normal thyroid tissue from the same patient.

FIG. 4. Comparison of VEGF-C expression, relative to GUS expression, among papillary thyroid cancers tissues, metastatic lymph nodes, and normal thyroid tissues from the same patients (*P < 0.05, ANOVA). Tumor, thyroid cancer tissue from the thyroid; Normal, normal thyroid tissue; LN, thyroid cancer tissue from metastatic lymph node (n = 5).